交換の回数にはカウントされません)．ただし，内容については双方の施設にて個人情報と施設情報がないことを必ずご確認くださいますようお願いします.」という新しいルールが公開されており，すべてのドナーの方に感謝の気持ちが伝わることを願っている．

6. おわりに

わが国では，非血縁者間 PBSCT の導入に関しては，他の国に比し大きく遅れたが，その間，学会のガイドラインのもと，血縁末梢血幹細胞採取が海外に比し，安全に実施され，また，全ドナーフォローアップ事業により，ドナーの短期および中長期の有害事象の情報が蓄積されてきた．この経験をもとに，導入にあたっては，“ゼロリスク”をめざし，ドナーの安全を十分に配慮したマニュアルの作成，施設認定，コーディネートシステムの構築が行われ，万全の体制で開始された．今後は，移植後の管理方法の確立，凍結の可否や医師の負担軽減，センター化など進行中の課題や解決しなければならない問題も多いが，患者およびドナー双方にとってより良い医療として発展することが望まれる．

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8．自家・同種骨髄・末梢血幹細胞の採取方法，ドナーの安全管理

はじめに

2010年10月よりわが国においても，非血縁末梢血幹細胞移植が開始され，非血縁ドナーにおいても骨髄採取，末梢血幹細胞採取の選択の幅が広がった。同種造血幹細胞移植は提供していただけるドナーの方がいなければ成り立たない治療であり，採取にかかわる担当医，麻酔科医，看護師，臨床工学士はゼロリスクを目指し，細心の注意を払って採取をする必要がある。

1．ドナーの準備

ドナーにはリスクと負担が伴うため，文書と口頭で十分説明し，文書による同意を得る必要がある。非血縁ドナーの場合は，十分なトレーニングを受けた骨髄移植推進財団(骨髄バンク)のコーディネーターによりドナーの自己決定に十分配慮が行われ，成人であっても本人の同意以外に家族の同意も必要である。血縁ドナーの場合も，院内コーディネーター（造血細胞移植臨床コーディネーター：HCTC）など，患者の主治医以外の者がHLA（human leukocyte antigen：ヒト白血球抗原）検査前から説明することで自由意思による提供に配慮する必要がある。HCTCがいる施設も増えてきており，日本造血細胞移植学会による研修会も2011年より開催されている。採取が決まれば，血算や生化学検査，血液凝固検査，感染症関連などの血液検査，尿検査に加えて胸部X線検査，心電図検査，呼吸機能検査（骨髄採取のみ）を行い，問題がないかをチェックしている（骨髄バンクでは，確認検査と術前健診で2回チェックしている）。

2．骨髄採取

1　同種骨髄採取

骨髄採取はThomasの原法[1]にもとづき，各移植施設で多少改変された方法で行われている。骨髄バンクでは骨髄採取マニュアル（第4版）[2]を作成し，細かい注意点も記述している。患者の体重から計算される標準採取量（骨髄バンクの場合は15 mL×患者体重）より採取予定量（血液型不一致の場合は赤血球除去のため20%程度割り増すこともある）を決定する。なお，非血縁ドナーの場合は，採取後の日常生活に支障がでないことを原則としており，ドナー体重とヘモグロビン量によって規定されているドナー上限量を超えての採取はできない（表）。

① 骨髄採取予定量から100～400 mLを引いた量を自己血として貯血する。自己血採血に際して鉄剤の経口投与を開始し，1回の貯血量は400 mLもしくは循環血液量（男性：体重×80 mL/kg，女性：体重×70 mL/kg）の10%以内（体重50 kg未満の場合）を上限として，1週1回以内で1～3回に分けて採血する（採取7日前までに完了する）。なお，採取バッグによって有効期限（CPD液の場合は21日，CPDA液の場合は35日）が異なっているので注意が必要である。ドナーの体調によっては採取が延期されることもあるため，有効期限の長いCPDA液が望ましい。自己血は必ず輸血部が管理する。

② 採取は2～4名の採取チームにより，気管内挿管による全身麻酔下，腹臥位で実施する。眼球圧迫・腕神経叢の過伸展・上肢尺骨神経圧迫等の予防に加えて，骨髄採取では採取針刺入圧増加による骨盤部の圧迫での外側大腿皮神経障害が報告されており，腹臥位の除圧枕の選択には注意を要し，蒲鉾型除圧枕は避ける。

③ 穿刺針はディスポーザブル，径は成人では11～13G（骨髄バンクでは13G, 2inchを推奨）を使用し，穿刺部位は両側の上後腸骨棘を中心に腸骨稜うしろ3分の1を原則とする（骨髄採取マニュアルに神経，血管の走行が詳細に示されている）[2]。

表　術前健診時のドナーHb値より算出したドナー上限量（骨髄バンク）

Hbが，男性は13.0 g/dL未満，女性は12.0 g/dL未満は採取中止
（女性のみ）
・Hb 12.5 g/dL未満は，ドナー体重1 kgあたり，12 mL/kg以下
・Hb 13.0 g/dL未満は，ドナー体重1 kgあたり，15 mL/kg以下
（男性，女性とも）
・Hb 13.5 g/dL未満は，ドナー体重1 kgあたり，15 mL/kg以下
・Hb 13.5 g/dL以上は，ドナー体重1 kgあたり，20 mL/kg以下

骨髄バンクでは，ドナーの日常生活に支障がでないように体重とヘモグロビン量によって上限量を定めている。（文献2より引用）

④骨髄液の凝固を防ぐために，希釈液としてヘパリンを加えた生理食塩水を準備し，採取するシリンジに 0.5 ～ 1.0 mL 程度入れておく。ヘパリンの濃度は施設によって多少異なるが，添付文書には輸血時 4 ～ 5 単位 /mL 総量と記載されており，骨髄バンクで行ったアンケートでは 10 単位 /mL 総量が最も多かった（ヘパリン 1 単位は血液 1 mL を凝固させない量として定義されている）。凝固しやすい場合は増量が必要となるが，患者に投与される総量が多い場合は，移植後，APTT (activated partial thromboplastin time：活性化部分トロンボプラスチン時間）が延長する。なお，歴史的には希釈液として TC199 や RPMI1640 などのメディウムが用いられているが，人体への投与が認められていないため，骨髄バンクでは推奨していない。

⑤採取部位を消毒した後，穿刺前にはタイムアウトを行い，採取予定量，自己血準備量，最大採取量などを全員で確認する。

⑥穿刺は上後腸骨棘を目標に垂直に行う。骨髄穿刺針を皮下組織に進め，骨膜にあたったところで，骨である手応えを確認し，骨髄穿刺針の頭を拇指球にあて，力を加え錐もみしながら進めていく。穿刺針の長さと腸骨の厚みを十分考慮し，穿刺の深さを調整する（穿刺場所によっては腸骨の厚みが薄い場所があるため，骨盤腔に貫通しないように注意が必要である[2]）。抵抗がなくなるか，もしくは 5 mm ～ 1 cm 程度挿入され，採取針が固定されていれば，内針を抜き，シリンジを固定して勢いよく陰圧をかけて単純吸引あるいはポンピングにより骨髄液を採取する。その際，シリンジが抜けないように固定しておく。1 回の吸引は末梢血の混入を防ぐため，3 ～ 10 mL の骨髄液を吸引する（Thomas の原法では 1 回 1 ～ 3 mL と記載されているが，最近の著書[3]では 5 ～ 10 mL と記載されている）。採取後，再び内針を挿入し，採取針を錐もみさせながら 5 mm 程度進め，さらに採取する。同一部位で数回採取した後，採取針を抜去し，ガーゼで圧迫する。同じ皮膚の針穴より採取針を入れ，少し皮膚をずらすようにして新しい骨の部分を穿刺する（角度をつけすぎると剥離骨折することもあるため注意が必要）。採取が始まれば，自己血の輸血を開始し，採取速度は 500 mL/30 分以下とする。採取困難なドナーの場合，長時間吸引を続けるとシリンジ内で骨髄液が凝固することがあるため，ときどき撹拌するなど工夫して採取を行う。なお，吸引後の骨髄液は骨髄内に戻してはならない。

⑦採取予定量の半分程度採取した時点で，細胞数を測定（自動血球測定装置で測定している場合は，赤芽球が除外されたカウントがでる場合があるため，必ず，有核赤血球を含めたカウントであるかを確認する）して予想をたて，最終的に患者体重あたり 3.0×10^8 個 /kg を目標とするが，2.0×10^8 個 /kg 以下でも生着は可能である。骨髄バンクの場合はドナー上限量を超えて採取してはならず，また，実出血量（採取骨髄量－自己血輸血量）も 400 mL 以下とする必要がある。

⑧ 採取した骨髄は，骨片や脂肪塊を除去するため，500 μm と 200 μm の 2 段階フィルター（多くの施設では市販のキット）[4,5]を用いて濾過し，回収したバッグはリークしないように 2 重にシールする。なお，バッグは破損なども考慮し，複数に分けて運搬する。

⑨ 血液型不一致の場合は，後述する血球分離装置を用いて，移植前に赤血球や血漿の除去を行う。

2 自家骨髄採取

同種骨髄採取と同様に行うが，骨髄液を濾過した後，赤血球を除去し，移植までの間は，末梢血幹細胞（後述）と同様に −80 ℃ 以下の超低温冷凍庫もしくは液体窒素に凍結保存しておき，移植時に解凍する。

3．末梢血幹細胞採取

1 同種末梢血幹細胞採取

日本造血細胞移植学会では，同種末梢血幹細胞移植のための健常人ドナーからの末梢血幹細胞動員・採取に関するガイドライン（第 3 版）[6]，骨髄バンクでは末梢血幹細胞採取マニュアル（暫定版）[2]を策定している。

① G-CSF（granulocyte colony-stimulating factor：顆粒球コロニー刺激因子）（フィルグラスチム 400 μg/m² またはレノグラスチム 10 μg/kg）を 1 日 1 回，または 2 分割で連日皮下注射する。白血球数が 50,000/μL を超えた場合は減量を検討し，75,000/μL を超えた場合は中止する。

② G-CSF 投与の 4 ～ 6 日目に血球分離装置を用いてアフェレーシスを実施する（7 日以後は CD34 陽性細胞は減少する）。G-CSF 投与 30 分後に，一過性に好中球は減少し，1 時間後に回復，その後増加を続け，4 ～ 8 時間後にピークとなる。CD34 陽性細胞についても同様の報告があり，アフェレーシス開始は G-CSF 投与後 4 時間以降が望ましい[7]。

③ 抗凝固薬として ACD-A 液を用いて血球分離装置の回路をプライミングし，採血および返血のための血管ルート（16 ～ 18 G 針）を確保する。両側肘静脈を用いるのが望ましいが，やむを得ない場合は大腿静脈などにブラッドアクセスを確保する。骨髄バンクでは，肘静脈の確保が難しいドナーは不適格としている。なお，数日間留置する場合は，透析用のダブルルーメン・カテーテルを使用する。

④ 採取のための処理血液量は 150 ～ 200 mL/kg あるいは循環血液量の 2 ～ 3 倍が一般的で，ACD-A 液を加えながら，血流速度 50 ～ 60 mL/ 分で体外循環を行い，必要な細胞を

濃縮する。アフェレーシスの所要時間は3時間前後となる。

⑤ フローサイトメトリーを用いてCD34陽性細胞を測定し，患者体重あたり2.0×10^6個を目標とするが，$1.0 \sim 2.0 \times 10^6$個でも生着は可能である。幹細胞の動員が不十分なpoor mobilizerも0.5%程度みられるが，あらかじめ予測することができないので，十分量が採取できたことを確認してから移植前処置治療を開始するため，凍結保存する場合もある。採取された幹細胞分画は凍害防止のためDMSO（dimethyl sulfoxide）とHES（hydroxyethyl starch）を調整したCP-1（極東工業製薬）[8]を添加し，プログラムフリーザーを用いて緩徐に凍結し液体窒素に保存する方法と，−80℃以下の超低温冷凍庫に直接保存する簡易法がある（1～5年間有効）。なお。非血縁の場合は骨髄同様凍結保存せずに移植を行う。

⑥ 凍結保存した場合は，移植の際に37～40℃の恒温槽で急速解凍し，輸注する。

2　自家末梢血幹細胞採取

自家末梢血幹細胞採取は，同種末梢血幹細胞採取と同様，G-CSFのみで行う場合もあるが，化学療法後の造血回復期にG-CSFを投与し，CD34陽性細胞をモニターし，採取を行う方が効率がよい。自動血球分析装置で未熟白血球を検出し，採取時期を推定することも検討されている[9]。原疾患の治療レジメだけではなく，自家末梢血幹細胞を動員する目的でシクロホスファミド（2 g/m²/日，2日間），シタラビン（2～3 g/m²/回，1日2回，2日間），エトポシド（500 mg/m²/日，3日間）などが用いられることもある。エトポシドは動員効率が高いが，点滴ルートの材質によっては亀裂が生じることがあるので注意が必要である。B細胞性腫瘍の場合，抗CD20抗体（リツキシマブ）を併用し，採取した幹細胞分画への腫瘍細胞の混入を防ぐために“*in vivo* purging”を行うこともある。

4．ドナーの安全管理

造血幹細胞を提供するドナーは，献血同様自由意思による健康なボランティアであるため，安全には十分配慮し，また，退院後すぐに社会生活に復帰できるように負担を最小限にする必要がある。過去に報告されたドナーの有害事象に関しては，骨髄バンク[10]および日本造血細胞移植学会[11]のホームページで詳細をみることができる。なお，採取に伴う合併症に対してドナー傷害保険が用意されている。非血縁ドナーはすべて骨髄バンクで手続きされる（費用負担は患者）が，血縁ドナーの場合は，日本造血細胞移植学会に登録した上で，掛金を患者・ドナー・家族のいずれかが自己負担する必要がある。

1　骨髄採取に伴う合併症

骨髄採取によるドナーの死亡例は，日本で腰椎麻酔下での呼吸停止1例，海外で過敏反応，心室細動，肺脂肪塞栓が各1例報告されており，100%安全であるとはいえないものの，最近の死亡例は報告されていない。骨髄バンクにおいても，後腹膜血腫や肺脂肪塞栓などの重篤な合併症も報告されているが[10]，その後回復している。しかし，採取中の神経圧迫による神経障害などに対して後遺障害保険が適用となった例（約0.1%）も報告されている。そのほか，椎間板ヘルニアの悪化や全身麻酔に伴う悪性高熱症も注意が必要である。また，自己血採血時にも，血管迷走神経反射（VVR）や内出血，正中神経損傷などの注意も必要である。

2　末梢血幹細胞採取に伴う合併症

末梢血幹細胞採取によるドナーの死亡例は，海外で脳血管障害3例，心不全，心筋梗塞，硬膜下出血，鎌状赤血球貧血クライシス，空気塞栓が各1例以外に，技術的な問題による合併症も含め合計12例報告されており，特に高齢者や動脈硬化などの合併症を有している例は注意を要する。初期には海外で白血球の急激な増加で脾臓が破裂した症例も報告された[12]。なお，わが国で末梢血幹細胞採取1年2カ月後に急性骨髄性白血病を発症した症例が報告されたが[13]，その後の調査では骨髄採取ドナーにも報告があり[14]，白血病の自然発生率を上回っていないことからも因果関係は不明である。なお，G-CSF投与により多くのドナーで骨痛が出現するが，一過性でアセトアミノフェン等の鎮痛薬で対応可能である。アフェレーシスの際には，抗凝固薬のACD-A液による低カルシウム血症に伴うしびれやテタニー症状を予防するためカルシウム製剤の点滴を行う場合が多い。また，VVRにより心停止に至る場合もあり，採取中は注意深いモニタリングが必要である。アフェレーシスにより血小板も採取されてしまうため，血小板が低下しすぎた場合は，自己多血小板血漿を作成してドナーに輸注することが望ましい。

3　骨髄採取と末梢血幹細胞採取の比較

骨髄採取と末梢血幹細胞採取はそれぞれ全く異なる手技で，有害事象も異なっており[14]，ドナーの負担を単純に比較することはできない。肉体的な侵襲は，穿刺回数にも依存するが，骨髄採取の方が強く，採取後，回復までの時間も長い[15]。また，採取時に尿道カテーテルを挿入する施設では，特に男性ドナーで尿道痛などの不快感が強い。一方，末梢血幹細胞採取では，覚醒状態で3時間あまりの採取に対する精神的なストレス，不安を訴えるドナーもある。また，肘静脈での採取が困難な場合，やむを得ず大腿静脈にブラッドアクセスを挿入する必要があるが，特に若い女性の場合にはストレスになると思われる。非血縁ドナーにおいて，骨髄採取と

末梢血幹細胞採取に関するSF-36を用いた観察研究が実施されているが，現時点では，患者の病状やドナーの希望を考慮し，採取方法を決定している。

5．採取後検診

採取終了後，穿刺部位や血液検査に問題がなければ退院可能で，骨髄バンクでは骨髄提供ドナーは採取後2日，末梢血幹細胞提供ドナーは採取日もしくは翌日（ブラッドアクセスを挿入した場合は翌日）としており，1カ月後を目処に採取後検診を行う。

6．ドナーサンクスカード運動

骨髄バンクのドナーは，見ず知らずの患者に骨髄や末梢血幹細胞を提供するボランティアである。この「命の贈り物」をもらった患者は，お互いのプライバシーを侵害しないように無記名でお礼の気持ちを手紙に託すことができる。しかし，様々な事情のため，実際にドナーにお礼の手紙が届くのは50%程度しかない。ドナーサンクスカード運動は，移植に携わるチームが移植医療を支えて下さっているドナーへ感謝の意を伝えようと始まった。採取施設のスタッフとして，提供していただいたドナーの方に，採取終了後サンクスカードをお渡しする。また，移植施設のスタッフとして，移植を受ける患者のために骨髄や末梢血幹細胞を提供してくださったドナーの方には，採取施設の主治医に手紙をことづけ，患者・ドナーのプライバシーを侵害しないようにドナーの方に渡してもらう。

平成23年9月15日発行の「MONTHLY JMDP」[16]に，「お手紙交換のルール変更について：移植施設の医師・医療スタッフのお立場でドナーの方にお手紙をご準備いただける場合は，当財団を通さずに採取施設のスタッフへ直接お渡しいただいて構いません（お手紙交換の回数にはカウントされません）。ただし，内容については双方の施設にて個人情報と施設情報がないことを必ずご確認くださいますようお願いします。」という新しいルールが公開されており，すべてのドナーの方に感謝の気持ちが伝わることを願っている。

（日野雅之・中前博久・中根孝彦・梅本由香里）

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Synthetic retinoid Am80 ameliorates chronic graft-versus-host disease by down-regulating Th1 and Th17

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Synthetic retinoid Am80 ameliorates chronic graft-versus-host disease by down-regulating Th1 and Th17

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Chronic GVHD (cGVHD) is a main cause of late death and morbidity after allogeneic hematopoietic cell transplantation, but its pathogenesis remains unclear. We investigated the roles of Th subsets in cGVHD with the use of a well-defined mouse model of cGVHD. In this model, development of cGVHD was associated with up-regulated Th1, Th2, and Th17 responses. Th1 and Th2 responses were up-regulated early after BM transplantation, followed by a subsequent up-regulation of Th17 cells. Significantly greater numbers of Th17 cells were infiltrated in the lung and liver from allogeneic recipients than those from syngeneic recipients. We then evaluated the roles of Th1 and Th17 in cGVHD with the use of IFN-γ–deficient and IL-17–deficient mice as donors. Infusion of IFN-$\gamma^{-/-}$ or IL-$17^{-/-}$ T cells attenuated cGVHD in the skin and salivary glands. Am80, a potent synthetic retinoid, regulated both Th1 and Th17 responses as well as TGF-β expression in the skin, resulting in an attenuation of cutaneous cGVHD. These results suggest that Th1 and Th17 contribute to the development of cGVHD and that targeting Th1 and Th17 may therefore represent a promising therapeutic strategy for preventing and treating cGVHD. (*Blood.* 2012; 119(1):285-295)

Introduction

GVHD is a result of immune attack of host tissues, such as the skin, gut, liver, and lung, by donor T cells in transplants.[1,2] On the basis of the differences in clinical manifestations and histopathology, GVHD can be divided into acute and chronic types. Chronic GVHD (cGVHD) is the main cause of late death and morbidity after allogeneic hematopoietic stem cell transplantation.[3-5] cGVHD often presents with clinical manifestations that resemble those observed in autoimmune diseases, such as systemic lupus erythematosus, Sjögren syndrome, lichen planus, and scleroderma. It has traditionally been assumed that the predominant cytokines produced during acute GVHD are Th1 cytokines, whereas those produced during cGVHD are Th2 cytokines. Although recent studies have suggested that cGVHD could be caused by cytokines secreted by Th1 cells,[6] Th17 cells,[7] or autoantibodies,[8] or both, the immune mechanisms leading to the development of cGVHD are not completely understood.

Th17 cells are a third subset of polarized effector T cells characterized by their expression of proinflammatory cytokine IL-17 and other cytokines.[9] IL-17 belongs to a family of 6 members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25), and IL-17F. Of these, IL-17A and IL-17F are the best characterized cytokines and form heterodimers. IL-17 plays an important role in the control and clearance of various pathogens.[9] In addition, Th17 cells have been implicated in allograft rejection of solid organs and several autoimmune diseases.[10,11] Although a number of studies have addressed how Th17 cells contribute to GVHD[12] and have reported that Th17 cells are sufficient but not necessary to induce acute GVHD,[13,14] the functional role of Th17 in cGVHD is unclear.

Retinoic acid, the active metabolite of vitamin A, has multiple effects on cell differentiation and survival by ligating the receptors from 2 families, retinoic acid receptors (RARs) and retinoid X receptors, each of which exists in multiple isoforms.[15] All-*trans*-retinoic acid (ATRA) has been reported to inhibit IFN-γ synthesis by Thl cells and to suppress the differentiation of Th17 cells by down-regulating the orphan nuclear receptor RORγt, a key regulator of Th17 differentiation.[16-19] Am80 is a novel RARα/β-specific synthetic retinoid that shows ~ 10-fold more potent biologic activity than ATRA by binding to RARα and RARβ but not to RARγ.[20] Am80 also inhibits IL-6 signaling[20,21] and reduces the severity and progression of inflammatory disease models.[20-23]

In the present study, we used the B10.D2 (H-2^d) into BALB/c (H-2^d) MHC-compatible, multiple minor histocompatibility Ag (miHA)–incompatible model of cGVHD to address the contribution of Th1/Th17 cells and the effects of retinoids on cGVHD with the use of IFN-$\gamma^{-/-}$ mice and IL-$17^{-/-}$ mice as donors. We also tested the hypothesis that the administration of Am80 ameliorates cGVHD by reducing the levels of Th1 and Th17 inflammatory cytokines and the fibrosis factor TGF-β.

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Methods

Mice

Female B10.D2 (H-2^d) mice were purchased from Japan SLC. BALB/c (H-2^d) recipient mice were purchased from Charles River Japan. IL-17A–deficient (IL-$17^{-/-}$) mice with the BALB/c background were generated previously.[24] IFN-γ–deficient (IFN-$\gamma^{-/-}$) mice were purchased from The Jackson Laboratory. IL-$17^{-/-}$ and IFN-$\gamma^{-/-}$ mice with the B10.D2 background were backcrossed for 8-10 generations from the original knockout mice. All experiments involving animals were performed according to the regulations of the Institutional Animal Care and Research Advisory Committee, Okayama University Advanced Science Research Center.

BM transplantation

Mice received transplants according to the standard protocols described previously.[25] Briefly, BALB/c mice received a single dose of 6.75 Gy x-ray total body irradiation. Recipient mice were injected with 2×10^6 spleen T cells and 8×10^6 T cell–depleted BM (TCD-BM) cells from B10.D2 donors. T-cell depletion and purification were performed with anti-CD90.2 Microbeads, pan T-cell isolation kit, and CD25 isolation kit and an AutoMACS system (Miltenyi Biotec) according to the manufacturer's instructions. Donor cells were injected intravenously into the recipients on day 0.

Evaluation of cGVHD

After BM transplantation (BMT), animals were weighed every 3 days and scored for skin manifestations of GVHD. The following scoring system was used[25]: healthy appearance, 0; skin lesions with alopecia < 1 cm^2 in area, 1; skin lesions with alopecia 1-2 cm^2 in area, 2; skin lesions with alopecia > 2 cm^2 in area, 3. In addition, animals were assigned 0.3 points each for skin disease (lesions or scaling) on the ears, tails, and paws. The minimum score was 0, and the maximum score was 3.9.

Tissue histopathology

Shaved skin from the interscapular region (~ 2 cm^2), the left lung, liver, and colon specimens of recipients were fixed in 10% formalin, embedded in paraffin, sectioned, mounted on slides, and stained with H&E. Slides were scored by a pathologist blind to experimental group (K.T.) on the basis of dermal fibrosis, fat loss, inflammation, epidermal interface changes, and follicular drop-out (0-2 for each category; the maximum score was 10).[25] Lung, liver, and colon slides were scored by a pathologist blind to the experimental group (T.T.). Lung slides were scored according to periluminal infiltrates, pneumonitis, and the extent of injury (0-3 for each category), and the maximum score was 9.[26] Liver slides were scored according to bile duct injury and inflammation (0-4 for each category), and the maximum score was 8.[27] Colon slides were scored according to crypt apoptosis and inflammation (0-4 for each category), and the maximum score was 8.[27]

Intracellular cytokine staining and cytokine analysis

Organs from mice were removed, processed into single-cell suspensions, and stimulated in vitro with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 100 ng/mL ionomycin (Sigma-Aldrich) at 37°C for 3 hours. Cells were then incubated with GolgiStop (BD PharMingen) for an additional 2 hours. mAbs conjugated to fluorescein isothiocyanate, phycoerythrin, peridinin-chlorophyll protein complexes, allophycocyanin, or Alexa Fluor 488 were used to assess the cell populations and were purchased from BD PharMingen or eBioscience. Cells were analyzed on a FACSCalibur flow cytometer with CellQuest software (both from Becton Dickinson) or MACS Quant flow cytometer (Miltenyi Biotec) with FlowJo software (TreeStar); both were housed in the Central Research Laboratory, Okayama University Medical School. Total peripheral lymph node (PLN) cells were adjusted to 1×10^6/mL in cultures. Supernatants were removed, and cytokine levels were measured with a BD Cytometric Bead Array (CBA) or by ELISA (R&D Systems) according to the respective manufacturer's protocol.

IFN-γ neutralization

Anti–mouse IFN-γ mAbs for in vivo experiments were prepared from mouse ascites from clones R4-6A2. Mice were treated intraperitoneally with anti–IFN-γ mAbs or rat IgG (160 μg/mouse; Sigma-Aldrich) on days 0, 5, 10, and 15 after BMT.

Administration of ATRA and Am80

Recipients were orally administered ATRA (200 μg/mouse; Wako), Am80 (1.0 mg/kg body weight; Nippon Shinyaku), or vehicle solutions daily from day 0.

Real-time RT-PCR

Total RNA was isolated from homogenized ear tissue with the use of an RNeasy mini kit (QIAGEN). cDNA synthesis was initiated by application of oligo dT primers and TaqMan Reverse Transcription Reagents (Applied Biosystems). Target cDNA levels were quantified by real-time PCR. The TaqMan Universal PCR Master Mix and the following Assay-on-Demand mouse gene-specific fluorescently labeled TaqMan MGB probes were used in an ABI Prism 5300 sequence detection system (Applied Biosystems): Mm01178820_m1 (TGF-β1). The mRNA expression of individual genes was normalized relative to GAPDH with the use of the equation $dCt = Ct_{target} - Ct_{GAPDH}$. The samples were obtained at room temperature using light microscopy (BX51; Olympus) with an objective lens (10×/0.40 NA, or 20×/0.70 NA; Olympus) and a camera (DP-70; Olympus). The images were acquired with image processing software (DP2-BSW Version 1.2; Olympus).

Statistical analyses

Group comparisons of skin cGVHD scores and pathology scores were performed using the Mann-Whitney U test or Kruskal-Wallis test. Cell populations, cytokine levels, mean weights, and gene expression data were analyzed with the unpaired 2-tailed Student t test. In all analyses, $P < .05$ was taken to indicate statistical significance.

Results

Th17 cells are increased in lymphoid organs during cGVHD development

We first assessed the kinetics of Th1/Th2/Th17 cytokine production of donor T cells generated during cGVHD. We used the most common cGVHD model: the MHC-compatible, multiple miHA-incompatible allogeneic BMT model (B10.D2 into BALB/c). Sublethally irradiated (6.75 Gy) BALB/c mice were transplanted with 2×10^6 B10.D2 spleen T cells and 8×10^6 B10.D2 TCD-BM cells. Ly9.1 was used as a marker to distinguish donors from recipients; B10.D2 and BALB/c are negative and positive for Ly9.1, respectively. Flow cytometric analysis of the spleens and PLNs on days 14 and 28 indicated that donor chimerism as determined by the negativity for Ly9.1 was $> 95\%$. The allogeneic recipients showed pathologic damage to the skin, salivary glands, lung, and liver, as reported previously (Figure 1A).[25,27] Cells isolated from PLNs were harvested on days 14 and 28 after BMT and analyzed for cytokine expression. In the early phase (day 14), IL-17^+ T cells were detected more frequently in the PLNs of recipients of syngeneic BMT, whereas in the late phase (day 28), IL-17^+ T cells in allogeneic recipients increased and were detected significantly more frequently than in syngeneic recipients (Figure 1B). We detected consistently higher percentages of donor T cells expressing IFN-γ and IL-13 in PLNs from allogeneic recipients than from syngeneic recipients (Figure 1B). Intracellular staining showed that most of the IL-17–producing cells were CD4$^+$ T cells (Figure 1C) and that IFN-γ/IL-17 double-positive cells (Th1/Th17

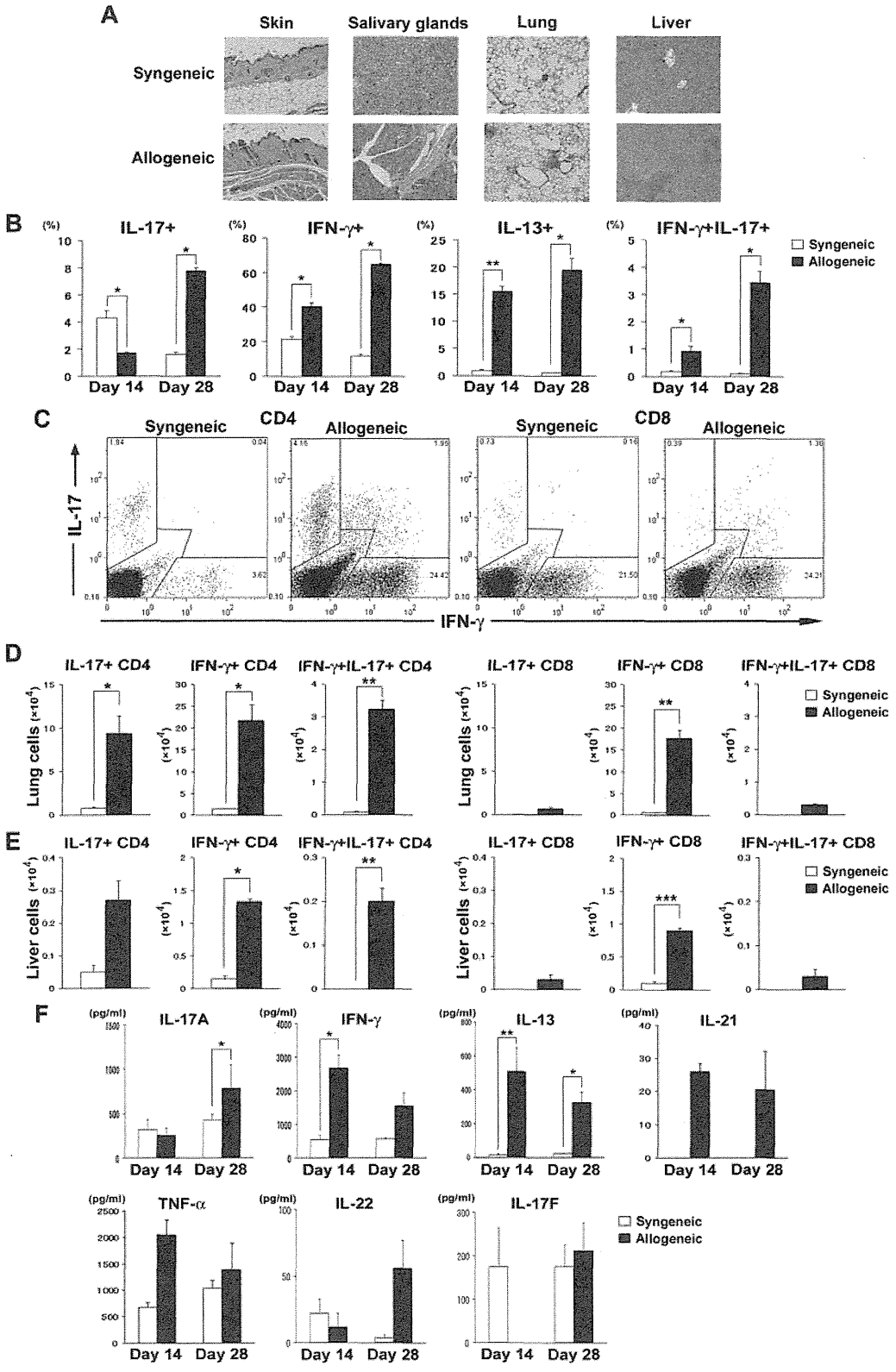

Figure 1. Th17 cells are increased in lymphoid organs during the late phase of cGVHD. Sublethally irradiated (6.75 Gy) BALB/c mice were transplanted with 2×10^6 spleen T cells plus 8×10^6 TCD-BM from WT B10.D2 mice (allogeneic group; black bars). The syngeneic group (white bars) received a transplant of the same dose of splenocytes and TCD-BM from BALB/c mice. (A) Histopathology of skin, salivary glands, lung, and liver of syngeneic and allogeneic recipients 35 days after BMT. (B) The percentages of donor-derived $CD3^+$ T cells expressing IL-17, IFN-γ, IL-13, and IFN-γ/IL-17 on days 14 and 28 are shown. (C) Representative staining for intracellular IFN-γ and IL-17 on $CD4^+$ and $CD8^+$ T cells on day 28 for syngeneic and allogeneic mice. (D-E) Absolute numbers of IL-17–, IFN-γ–, and IFN-γ/IL-17–producing $CD4^+$ and $CD8^+$ T cells in recipient lung (D) and liver (E). (F) PLN cells from syngeneic and allogeneic recipients on days 14 and 28 were stimulated with PMA and ionomycin in vitro. Five hours later, the supernatants were collected to determine cytokine levels by ELISA or CBA. Graphs indicate the levels of cytokines secreted per 1×10^6 total stimulated PLN cells. Three to 6 mice per group were used. The means (± SE) of each group are shown. Data are from 1 representative of ≥ 2 independent experiments. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .005$.

cells) were exclusively detected in allogeneic recipients (Figure 1B-C). As allogeneic recipients developed GVHD-induced lymphopenia on day 28; absolute numbers of IFN-γ^+ T and IL-17^+ T cells in PLNs from allogeneic recipients were not greater than those from syngeneic recipients (IFN-γ^+ T, $51.8 \pm 17.5 \times 10^4$ vs $49.4 \pm 4.2 \times 10^4$, $P = .92$; IL-17^+ T, $5.9 \pm 2.2 \times 10^4$ vs $6.9 \pm 0.59 \times 10^4$, $P = .16$). Numbers of Th1 and Th17 cells from allogeneic recipients were significantly greater than those from syngeneic recipients in the lung (Figure 1D) and liver (Figure 1E). Cells isolated from PLNs of allogeneic recipients secreted significantly greater amounts of IL-17, IFN-γ, and IL-13 after stimulation with PMA and ionomycin (Figure 1F) or without stimulation (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). These cytokine levels were also elevated in serum from allogeneic recipients 28 days after BMT (supplemental Figure 2). To confirm that our observations were not strain dependent or model dependent, we performed similar experiments in the DBA/2 into BALB/c model of cGVHD. We confirmed the up-regulated Th1 and Th17 responses in this model (supplemental Figure 3).

IL-$17^{-/-}$ donor T cells ameliorate cGVHD

We next used IL-$17^{-/-}$ mice with the B10.D2 background as donors to evaluate whether Th17 contributes to cGVHD. On transfer of IL-$17^{-/-}$ B10.D2 donor T cells into allogeneic BMT models, weight loss was mild and fur loss was clearly ameliorated in comparison to that seen in recipients of wild-type (WT) T cells (Figure 2A-B). Clinical cGVHD severity was assessed with a standard scoring system (see "Methods"). Allogeneic IL-$17^{-/-}$ BMT recipients showed significantly less skin cGVHD than WT controls ($P < .05$; Figure 2C). Histopathologic examination of the skin showed significantly reduced cGVHD pathology in recipients of IL-$17^{-/-}$ donors (3.17 ± 1.09 vs 8.50 ± 0.84; $P < .01$; Figure 2D). A dry mouth is one of the distinctive features of cGVHD, and lymphocytic inflammation, fibrosis, and atrophy of acinar tissue were observed in the salivary glands of WT BMT recipients. Histopathologic examination of the salivary glands showed reduced cGVHD pathology in the recipients of IL-$17^{-/-}$ donors (Figure 2E). Atrophy of the salivary glands as determined by their size was significantly reduced in recipients of IL-$17^{-/-}$ donors (4.21 ± 0.13 vs 3.54 ± 0.11; $P < .01$; Figure 2E). No significant differences were observed in pathology scores of the lung, liver, or colon between recipients of IL-$17^{-/-}$ and WT donors (lung, 2.6 ± 1.04 vs 0.8 ± 0.44, $P = .19$; liver, 1.5 ± 0.87 vs 1.83 ± 0.37, $P = .75$; colon, 1.6 ± 0.36 vs 2.8 ± 0.33, $P = .06$). Thus, IL-$17^{-/-}$ BMT recipients showed less cGVHD in the skin and salivary glands than did the WT controls. Flow cytometric analysis of the PLNs in the early phase (day 14) showed no differences in frequency of IFN-g^+ cells between IL-$17^{-/-}$ and WT recipients, whereas recipients of IL-$17^{-/-}$ showed fewer IFN-γ^+ cells in the late phase (day 35, $4.3\% \pm 0.8\%$ vs $18.9\% \pm 3.5\%$; $P = .01$; Figure 2F). As allogeneic WT recipients developed more severe GVHD-induced lymphopenia on day 35 than IL-$17^{-/-}$ recipients, absolute numbers of IFN-g^+ cells in PLNs from allogeneic WT recipients were not greater than those from IL-$17^{-/-}$ recipients (IFN-γ^+ T cells, $6.08 \pm 0.87 \times 10^4$ vs $4.83 \pm 1.23 \times 10^4$; $P = .48$). As expected, IFN-γ/IL-17 double-positive cells were not detected in recipients of IL-$17^{-/-}$ donors on days 14 and 35 (Figure 2G-H). No differences were observed in the IL-13^+ cells or Foxp3^+ cells between the groups (data not shown). These data suggest that donor IL-17 contributes to the pathogenesis of cGVHD.

Donor Th1 differentiation is responsible for the development of cGVHD

To test whether donor Th1 differentiation is responsible for cGVHD, we used IFN-$\gamma^{-/-}$ mice with the B10.D2 background as donors. BMT from IFN-$\gamma^{-/-}$ donors compared with WT donors significantly improved the clinical cGVHD score ($P < .05$; Figure 3A). Histopathologic examination of the skin showed significantly reduced cGVHD pathology in recipients of IFN-$\gamma^{-/-}$ donors (4.75 ± 0.54 vs 7.80 ± 0.52; $P = .02$; Figure 3B). Salivary gland atrophy was also reduced in recipients of IFN-$\gamma^{-/-}$ donors (3.81 ± 0.05 vs 2.87 ± 0.19; $P < .05$; Figure 3C). No significant differences were observed in pathology scores of the lung, liver, or colon between recipients of IFN-$\gamma^{-/-}$ and WT donors (lung, 2.4 ± 0.61 vs 3.2 ± 0.52, $P = .4$; Figure 3B; liver, 1.0 ± 0.4 vs 1.6 ± 0.32, $P = .21$; colon, 0.75 ± 0.21 vs 1.6 ± 0.67, $P = .36$). Intracellular staining of PLNs showed no differences in IL-13– or IL-17–producing cells between IFN-$\gamma^{-/-}$ and WT recipients (data not shown), although significantly greater numbers of Foxp3^+ cells were detected in the IFN-$\gamma^{-/-}$ recipients (day 35; $P < .05$; Figure 3D). To examine whether an increase in numbers of Treg cells was responsible for the reduced cGVHD in the absence of donor IFN-$\gamma^{-/-}$, mice were injected with whole T cells or CD25-depleted T cells from donors. As shown in Figure 3E, depletion of CD25^+ cells from the donor inoculum exacerbated skin scores ($P < .05$). However, CD25-depleted T cells from IFN-$\gamma^{-/-}$ mice caused less severe skin GVHD than those from WT mice ($P < .05$). These findings suggest that IFN-γ contributes to the pathogenesis of cGVHD by both Treg-independent and -dependent pathways. Next, we evaluated the role of IFN-γ in the development of skin cGVHD by administering anti–IFN-γ mAbs to recipients of WT or IL-$17^{-/-}$ donors. Anti–IFN-γ mAb treatment significantly reduced skin scores and pathology scores in recipients of WT donors (Figure 3F-G). Recipients of IL-$17^{-/-}$ donors again showed reduced skin scores, and treatment with anti–IFN-γ mAbs further reduced skin scores (Figure 3H). These findings suggest that IFN-γ contributes to cGVHD pathogenesis.

Am80 inhibits donor Th1 and Th17 cells both in vitro and in vivo

ATRA has been reported to suppress the differentiation of Th17 cells with a reciprocal induction of Treg cells.[28] Am80, a novel RARα/β-specific synthetic retinoid, has a biologic activity ~ 10 times more potent than that of ATRA[20] and directly inhibits Th1 cytokine production.[20,22,29] Therefore, we hypothesized that ATRA or Am80 down-regulates both Th1 and Th17 differentiation in donor T cells, resulting in attenuation of cGVHD. To clarify whether retinoids directly inhibit the production of cytokines, PLNs were isolated from mice 14 days after allogeneic BMT and cultured with Am80 for 24 hours to determine cytokine production. Am80 inhibited IFN-γ (Figure 4A) and IL-17 (Figure 4B) production in a dose-dependent manner. Next, BMT recipients were orally administered Am80 at a dose of 1.0 mg/kg of body weight or vehicle daily from day 0 of BMT, and cytokine expression was assessed in PLNs harvested on day 35. We detected significantly fewer IFN-γ^+ T cells in Am80-administered recipients (Figure 4C). In addition, PLNs from Am80-treated recipients produced significantly less IFN-γ after stimulation with PMA and ionomycin ($P < .01$; Figure 4D). No difference was observed in the percentage of IL-17–producing donor cells, although PLN cells from Am80-treated recipients produced significantly less IL-17 ($P < .05$) and IL-21 ($P < .01$) after stimulation with PMA and ionomycin (Figure 4D). Taken together, these data suggest that Am80 down-regulates both Th1 and Th17 cells in vitro and in vivo.

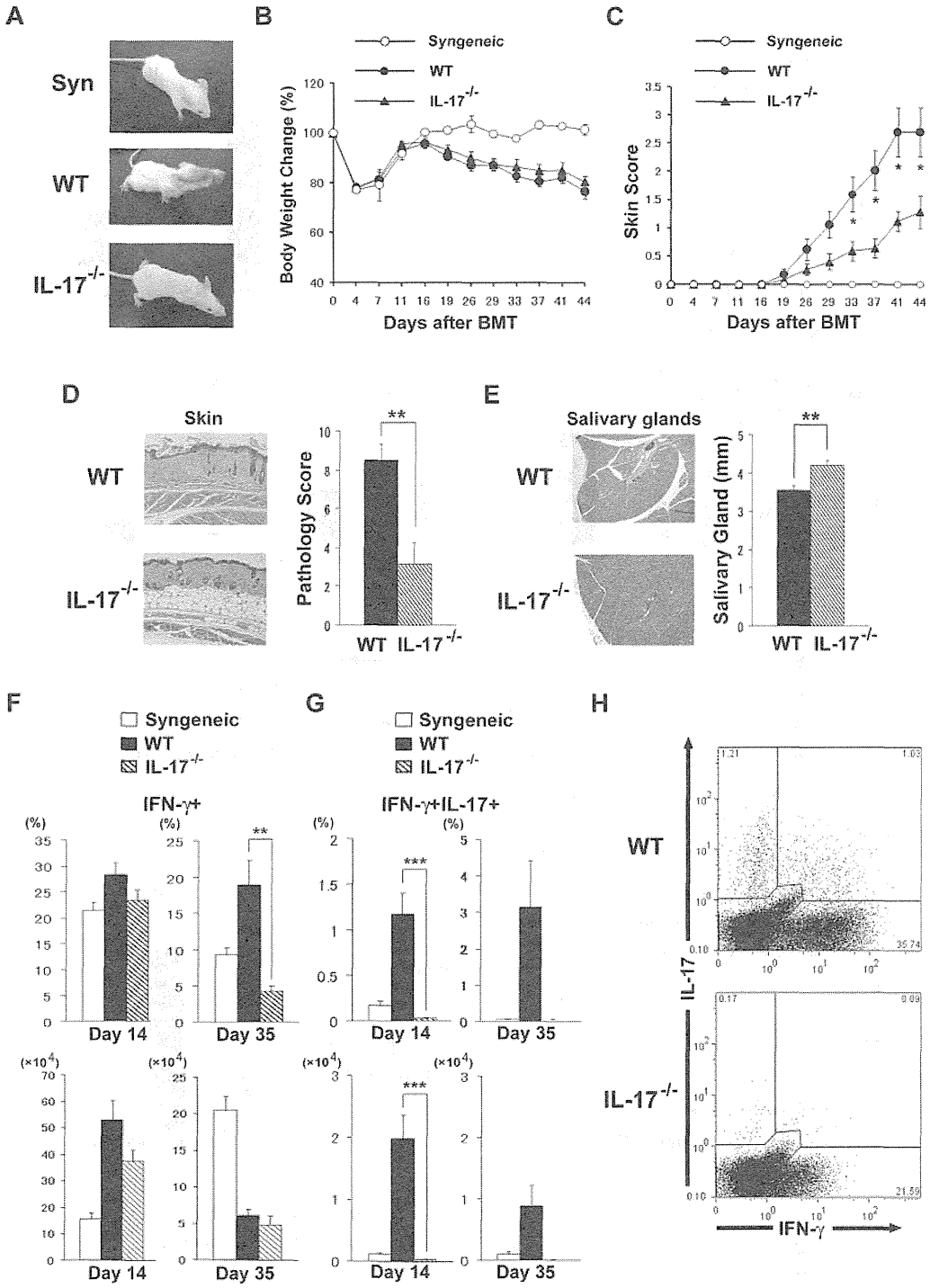

Figure 2. IL-17$^{-/-}$ donor T cells ameliorate cGVHD. Sublethally irradiated BALB/c recipients were transplanted from WT, IL-17$^{-/-}$ B10.D2, or syngeneic BALB/c donors. (A) Gross observation of the skin lesions from recipients of syngeneic, WT, and IL-17$^{-/-}$ donors 28 days after BMT. The recipients were analyzed for body weight (B) and cGVHD skin scores (C); data from 2 independent experiments were combined (n = 14 per group). Pathology score of skin (D) and the longest diameter of the salivary gland (E) on day 35 of BMT are shown. Four to 6 recipients were examined in each group. (F-G) PLN cells of the recipients of syngeneic (white bar), WT (black bar), or IL-17$^{-/-}$ (striped bar) donors were stained for intracellular IFN-γ and IL-17 on days 14 and 35 after BMT. The percentages and absolute numbers of IFN-γ$^+$ cells (F) and IFN-γ$^+$/IL-17$^+$ cells (G) are shown. Data from 2 replicated experiments were combined (n = 6-11 per group). (H) Representative staining for intracellular IFN-γ and IL-17 on CD4$^+$ T cells of WT or IL-17$^{-/-}$ mice on day 35 is shown. Data represent the means ± SEs. *$P \leq .05$, **$P \leq .01$, and ***$P \leq .001$.

Administration of Am80 ameliorates cGVHD

Next, we examined whether ATRA or Am80 can down-regulate cGVHD. BALB/c recipients were orally administered ATRA (200 μg/mouse) or Am80 from day 0 of BMT. We found that ATRA tended to decrease the clinical cGVHD score (Figure 5A), whereas Am80 significantly ameliorated the clinical score compared with controls ($P = .01$; Figure 5B). Histopathologic examination of the skin on day 16 showed significantly reduced cGVHD damage in Am80-treated animals (day 16, 4.8 ± 0.4 vs 7.4 ± 0.4; $P < .01$; Figure 5C), No differences were observed in pathology scores of the lung, liver, or colon between the 2 groups (Figure 5C). Because it has been reported that Am80 can induce Treg cells,[29] we

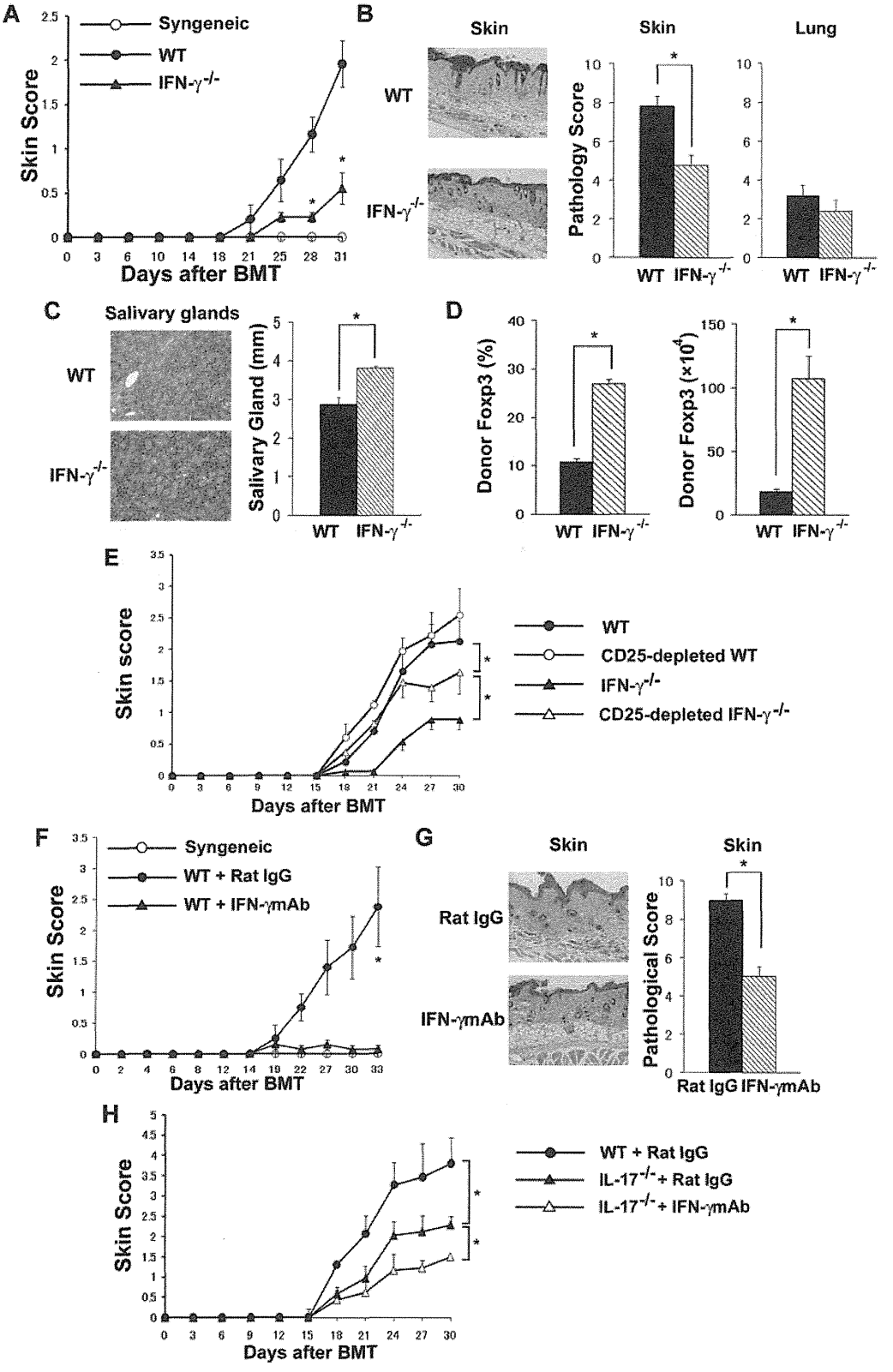

Figure 3. Donor Th1 differentiation and IFN-γ production are responsible for exacerbated cGVHD. (A-D) Sublethally irradiated BALB/c recipients were transplanted from WT or IFN-$\gamma^{-/-}$ B10.D2 donors. Clinical skin cGVHD scores (A), pathology score of skin and lung (B), and the longest diameter of the salivary gland (C) on day 35 after BMT are shown. Four to 6 recipients were examined in each group. Data are from 1 representative of 3 independent experiments. (D) PLN cells of the recipients on day 35 were stained for intracellular Foxp3. The percentages and the absolute number of $CD4^+Foxp3^+$ Treg cells are shown. Four to 6 recipients were examined in each group. Data are from 1 representative of 2 independent experiments. (E) Sublethally irradiated BALB/c recipients were transplanted 8×10^6 TCD-BM cells plus 2×10^6 total spleen T cells or CD25-depleted T cells from WT or IFN-$\gamma^{-/-}$ B10.D2 donors. The skin cGVHD scores are shown ($n = 6$ per group). Data are from 1 representative of ≥ 2 independent experiments. (F-H) Sublethally irradiated BALB/c recipients were transplanted from WT or IL-$17^{-/-}$ B10.D2 donors. The recipients were injected with anti–IFN-γ mAbs or rat IgG (160 μg/mouse) on days 0, 5, 10, and 15 after BMT and were assessed for the clinical signs of cGVHD every 3 days. The clinical skin cGVHD scores (F), histopathology, and pathology score of the skin (G) on day 35 of BMT from WT donors. Four mice per group were used. Data are from 1 representative of ≥ 2 repeated experiments. (H) The clinical skin cGVHD scores after BMT from WT or IL-$17^{-/-}$ donors are shown. Six mice per group were used. Data are from 1 representative of 2 independent experiments. The means (± SEs) of each group are shown; $^*P < .05$.

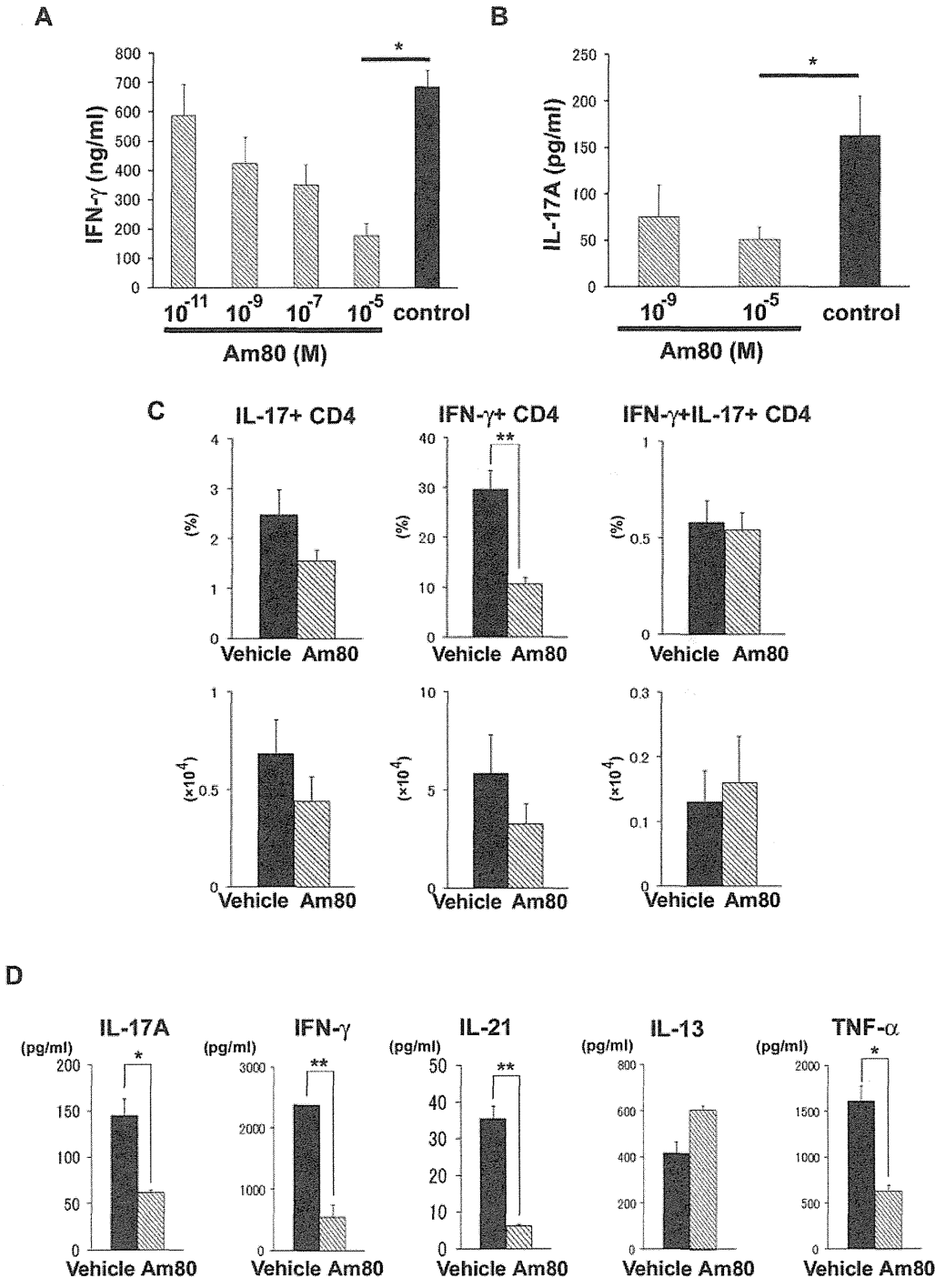

Figure 4. Am80 inhibits donor Th1 and Th17 cells in vitro and in vivo. Sublethally irradiated BALB/c recipients were transplanted from WT B10.D2 donors. (A-B) PLN cells from recipients (n = 3-6 per group) on day 14 were treated with Am80 or vehicle solution for 24 hours, the supernatants were collected, and ELISA was performed to determine the cytokine levels. Graphs represent the levels of cytokines secreted per 1×10^6 whole stimulated PLN cells. The data are from 1 representative of ≥ 3 independent experiments. (C-D) After BMT, recipients (n = 4-6 per group) were administered oral Am80 (1.0 mg/kg of body weight) or vehicle solution daily from day 0. PLNs of the recipients were stained for intracellular IFN-γ and IL-17. (C) The percentage and absolute number of IFN-γ$^+$ and IL-17$^+$–producing CD4$^+$ T cells. Data are from 1 representative of ≥ 2 repeated experiments. (D) PLN cells from recipients (n = 3-6 per group) treated with Am80 or vehicle on day 16 were stimulated with PMA and ionomycin. Five hours later, the supernatants were collected to determine cytokine levels by CBA. Graphs represent the levels of cytokines secreted per 1×10^6 whole stimulated PLN cells. The data are from 1 representative of ≥ 3 independent experiments. The means (± SEs) of each group are shown; $^*P < .05$ and $^{**}P < .01$.

quantified the frequency of Foxp3-expressing CD4$^+$ T cells in the PLNs after BMT. Recipients administered Am80 showed a decreased frequency of Foxp3$^+$ cells (day 17, 12.3% ± 2.5% vs 23.5% ± 2.6%; P = .02; Figure 5D). Foxp3 mRNA expression of the target organ (the ear) was also decreased in the Am80 recipients (data not shown). To confirm that the effects of Am80 are independent of Treg cells, mice were injected with whole T cells or CD25-depleted T cells from donors. As shown in Figure 5E, depletion of CD25$^+$ cells from the donor inoculum did not exacerbate skin cGVHD in Am80-treated mice, thus suggesting that the effects of Am80 treatment are not associated with Treg cells.

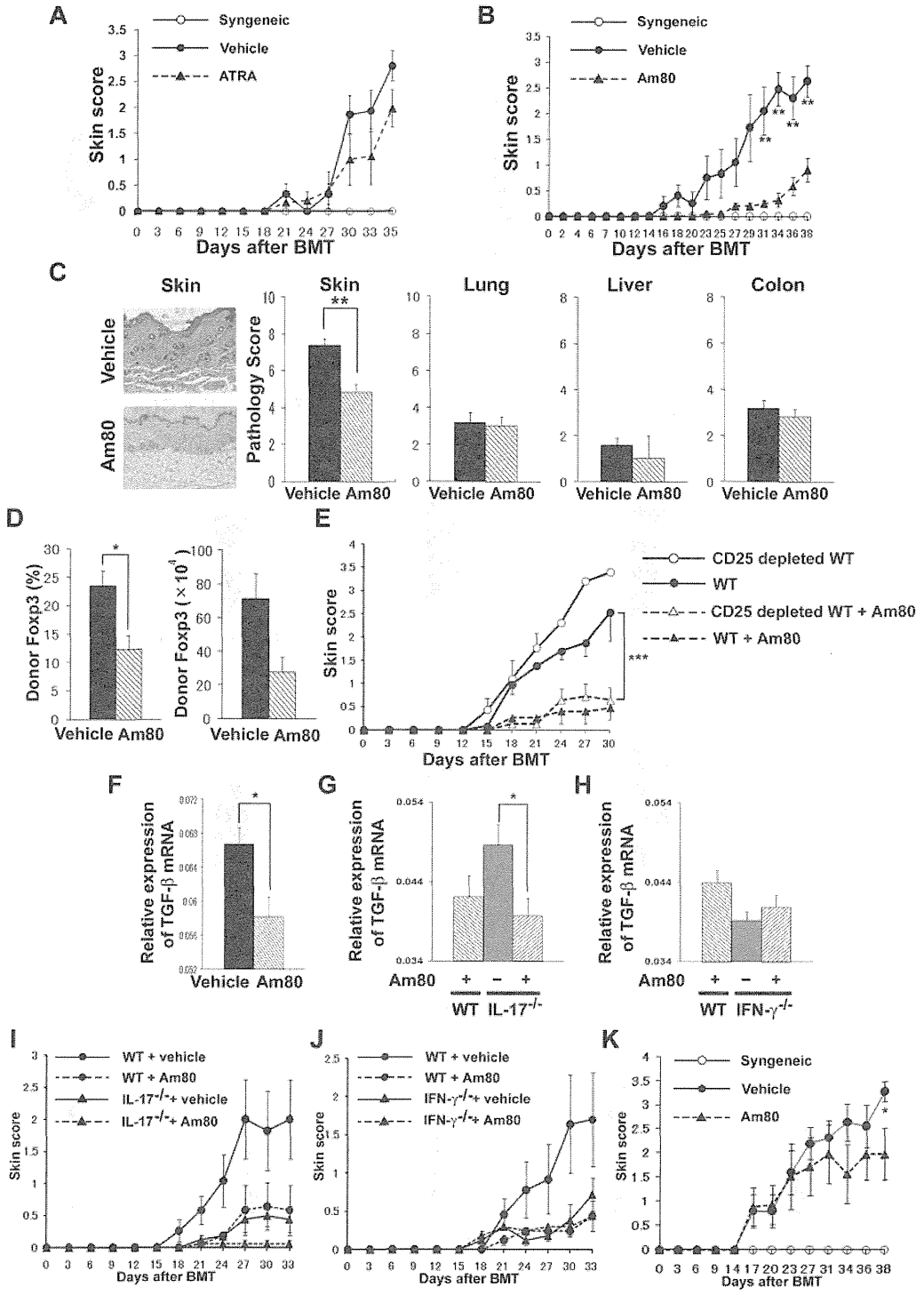

Figure 5. Administration of Am80 ameliorates cGVHD. (A-D) Sublethally irradiated BALB/c recipients were transplanted from WT B10.D2 donors. The recipients received daily administration of ATRA (200 μg/mouse; A), Am80 (1.0 mg/kg of body weight; B), or vehicle solution orally after BMT and were assessed for clinical signs of cGVHD every 3 days. The skin cGVHD scores are shown. (C) Representative histopathology of skin and pathology score of skin, lung, liver, and colon in each group (n = 5-6 per group) on day 16 after BMT are shown. (D) PLN cells of the recipients on day 16 were stained for intracellular Foxp3. The percentages and absolute numbers of $CD4^+Foxp3^+$ Treg cells are shown. Data are from 1 representative of ≥ 2 independent experiments. (E) Sublethally irradiated BALB/c recipients were transplanted with 8×10^6 TCD-BM cells plus 2×10^6 total spleen T cells or CD25-depleted T cells from WT or IFN-$\gamma^{-/-}$ B10.D2 donors. After BMT, recipients were given Am80 or vehicle solution. The skin cGVHD scores are shown. There were 6 recipients in each group; the data are from 1 representative of ≥ 2 independent experiments. (F-K) Sublethally irradiated BALB/c recipients were transplanted from WT (F), IL-$17^{-/-}$ (G), and IFN-$\gamma^{-/-}$ (H) donors. After BMT, recipients were given Am80 or vehicle solution. TGF-β mRNA expression in the ears on day 35 after BMT (F-H) and skin cGVHD scores (I-J) are shown. Data are from 1 representative of ≥ 2 independent experiments (n = 5 per group). (K) The skin cGVHD scores of BMT recipients treated with Am80 or vehicle solution orally daily after day 21 of BMT; data from 3 independent experiments were combined (n = 12-14 per group). $^*P < .05$, $^{**}P < .01$, and $^{***}P < .005$.

TGF-β is a critical mediator of fibrosis in cGVHD skin lesions.[30] TGF-β mRNA expression was decreased in the ear of the Am80 recipients (day 17, $P = .02$; Figure 5F). We then assessed TGF-β mRNA expression in recipients of IL-$17^{-/-}$ or IFN-$\gamma^{-/-}$

donors treated with Am80. Am80 further reduced skin scores and TGF-β expression in recipients of IL-17$^{-/-}$ donors (Figure 5G-I) but not in recipients of IFN-γ$^{-/-}$ donors (Figure 5H,J). These results suggest that the effects of Am80 are more dependent on IFN-γ than on IL-17.

Finally, we examined whether Am80 could be used for the treatment of cGVHD. Am80 was orally administered to mice from day 21 of BMT, when mice had developed clinical signs of cGVHD. Am80 significantly improved clinical scores ($P = .016$; Figure 5K).

Discussion

The results of the present study showed that Th1 and Th17 cells contribute to cGVHD with the use of a MHC-compatible, miHA-incompatible model of cGVHD. In addition, we demonstrated that Am80 down-regulates both Th1 and Th17 cells in vitro and in vivo, resulting in attenuation of cGVHD.

For many years, the best defined subsets of effector T cells of the CD4$^+$ Th lineage were the Th1 and Th2 cells. A third subset of CD4$^+$ effector cells was identified and named Th17 cells, because the signature cytokine that they produce is IL-17.[31] Although the role of Th17 in acute GVHD has been evaluated by several groups with inconsistent results,[32-35] few studies have addressed the role of Th17 in cGVHD. Initially, cGVHD was hypothesized to be a Th2-mediated disease on the basis of the results in a nonirradiated P→F1 model of cGVHD. cGVHD in this model is mediated by host B-cell autoantibody production stimulated by donor Th2 cells. Th1 polarization of donor T cells activates donor CD8$^+$ CTLs to kill host B cells, resulting in amelioration of cGVHD.[36] However, the relevance of this model is unclear in clinical BMT in which host B cells are eliminated by conditioning. Such different effector mechanisms between the models may be associated with distinct requirement of Th subsets for cGVHD between the studies. In the present study, we assessed the kinetics of Th1, Th2, and Th17 cells during the development of cGVHD in the B10.D2→BALB/c model. Th1 and Th2 responses were up-regulated early after BMT, followed by a subsequent up-regulation of Th17 cells. Significantly greater numbers of Th17 cells were detected in the lung and liver from allogeneic recipients than in those from syngeneic recipients. We then evaluated the role of Th17 in cGVHD with the use of IL-17$^{-/-}$ mice as several groups had used,[32-34,37,38] although interpretation of the results deserves caution because the Th17 lineage is uniquely regulated by RORγt,[13,14] and other cytokines such as IL-21 and IL-22 produced by Th17 cells may also contribute to Th17-mediated GVHD. On transfer of IL-17$^{-/-}$ B10.D2 donor T cells, cGVHD was significantly ameliorated compared with that in recipients of WT T cells, suggesting that Th17 contributes to cGVHD in this model. In particular, Th17 plays a significant role in skin cGVHD. This agrees with the recent observation by Hill et al[37] that donor pretreatment with G-CSF induces Th17 differentiation of donor T cells and induces skin GVHD after peripheral blood stem cell transplantation. In an adoptive transfer model of autoimmune cGVHD, Th17 cells infiltrated target tissues.[39] However, a subsequent study showed the absence of donor Th17 cells did not abrogate GVHD pathology,[38] in contrast to our results. In the absence of donor IL-17, Th1 responses were preserved in that study but were reduced in our study. Such difference in Th1 responses may produce different outcomes between the studies. In mouse models of acute GVHD, Yi et al showed enhanced Th1 differentiation of donor T cells by increased production of IL-12 from dendritic cells in the absence of IL-17.[33] By contrast, Kappel et al showed reduced numbers of IFN-γ–positive CD4$^+$ T cells and IFN-γ secretion in culture in the absence of IL-17.[34] These results together with our results suggest that IL-17 may induce IFN-γ, although such a hierarchy of Th1/Th17 pathways may be context or model dependent or both and will need to be studied in the future. Nonetheless, it should be noted that cGVHD still developed in the absence of donor IL-17 cells in our study. Taken together, it is probable that Th17 is not an absolute requirement for cGVHD, and either Th1 or Th17 is sufficient to cause cGVHD.

We demonstrated that IFN-γ$^{-/-}$ donor mice and injecting anti–IFN-γ mAb ameliorated cGVHD. Thus, Th1 and Th17 responses play a pathogenic role in cGVHD in this model. These results were consistent with a recent study reporting that cGVHD is mediated by Th1 and Th17 responses because of the progressive loss of CD4$^+$CD25$^+$Foxp3$^+$ T cells during acute GVHD in mice.[39] These results were also consistent with clinical studies showing that Th1 cells and Th17 cells increased in patients with active cGVHD.[40-43] Increased transcription of IFN-γ has also been detected in the affected skin and oral mucosa of patients with cGVHD.[41,44] In this study, we found no differences in Th17 cells between IFN-γ$^{-/-}$ and WT recipients, although significantly greater numbers of Treg cells were detected in IFN-γ$^{-/-}$ recipients. CD25-depleted T cells from IFN-γ$^{-/-}$ mice induced more severe skin cGVHD compared with CD25-replete IFN-γ$^{-/-}$ T cells, but still less severe cGVHD compared with CD25-depleted T cells from WT mice (Figure 3E), suggesting that IFN-γ contributes to the pathogenesis of cGVHD by both Treg-independent and -dependent pathways. Neutralization of IFN-γ ameliorated cGVHD in the absence of donor IL-17 (Figure 3H), suggesting again that both Th1 and Th17 responses contribute to the pathogenesis of cGVHD.

We found that donor-derived Th17 cells were generated in recipients of syngeneic transplantation in addition to allogeneic transplantation. However, the kinetics of Th17 development differed between the syngeneic and allogeneic settings; Th17 cells developed in the early phase after syngeneic transplantation. Kappel et al speculated that Th17 development may be the result of increased immune reconstitution of syngeneic hosts compared with allogeneic hosts with GVHD.[34] We additionally identified a population of donor-derived IFN-γ$^+$IL-17$^+$ cells after allogeneic BMT. It has been shown that a subset of IL-17–producing cells can also produce IFN-γ in vivo.[34,45] Such CD4$^+$IFN-γ$^+$IL-17$^+$ T cells have been postulated to play a causative role in the pathogenesis of experimental autoimmune encephalomyelitis (EAE).[46] IFN-γ$^+$IL-17$^+$ cells were only detected after allogeneic BMT, but not after syngeneic BMT, suggesting that this population is generated by allogeneic stimulation, but not because of lymphopenia-induced proliferation. Further investigations are required to clarify the difference in function between IL-17 single-positive and IFN-γ/IL-17 double-positive cells.

ATRA suppresses Th17 differentiation and effector function by RARα signaling,[18] but ATRA can also bind to RARβ and RARγ, which can form a variety of homodimers and heterodimers with 3 retinoid X receptors.[15] Nonselective receptor binding is thought to be a main cause of the side effects associated with the administration of ATRA and other pan-RAR agonists. Am80 is a synthetic RAR agonist that shows high affinity to RARα/β. In addition to a greater specificity for RARα, Am80 offers several other advantages over ATRA as a therapeutic agent, including less toxicity, greater stability, fewer potential side effects, and superior bioavailability. Am80 is effective in autoimmune disease models of collagen-induced arthritis,[20,47] EAE,[21,29] 2,4-dinitrofluorobenzene–

induced contact dermatitis,[22] and atherosclerosis.[23] Because retinoids can down-regulate Th1 and Th17 cells and can ameliorate autoimmune diseases, we hypothesized that these retinoids would attenuate cGVHD. We demonstrated that Am80 down-regulated Th1 and Th17 differentiation of donor T cells in BALB/c recipients of B10.D2 donors, resulting in reduced cGVHD. Our results suggest that combined blockade of Th1 and Th17 responses may represent a promising strategy to prevent or treat cGVHD, as has been suggested for acute and chronic GVHD.[32,39,48] Most recently, Yu et al used mice deficient for both T-bet and RORγt as T-cell donors and clearly showed that blockade of both Th1 and Th17 differentiation is required to prevent acute GVHD.[14] In addition, TGF-β mRNA expression in the skin decreased in the Am80 recipients of WT and IL-17$^{-/-}$ but not IFN-γ$^{-/-}$ donors. These results suggest that Am80 down-regulates TGF-β and that this effect is more dependent on IFN-γ than on IL-17. Unexpectedly, those recipients administered Am80 had a significantly lower frequency of Foxp3^{+} cells. These results differ from those of in vitro studies performed by Mucida et al,[28] in which retinoic acids were shown to be capable of inhibiting the IL-6–driven induction of Th17 cells and to promote Treg cell differentiation. Thus, retinoic acids enhance Treg differentiation and inhibit both Th17 and Th1 in vitro; however, the effects of retinoids may be more complex in vivo, because retinoids can affect not only T cells but also other immunoregulatory cells. For example, previous in vivo studies reported that Am80 suppressed Treg cells in experimental models of EAE[29] and collagen-induced arthritis,[47] similar to our study. In our study, Am80 suppressed TGF-β expression, a key cytokine in Treg development, which may have resulted in the suppression of Treg.

In conclusion, both Th1 and Th17 contribute to the development of cGVHD. Am80 down-regulates TGF-β and also regulates both Th1 and Th17 cells in vitro and in vivo, resulting in attenuation of cGVHD. Thus, administration of Am80, which is currently available as medication for acute promyelocytic leukemia in Japan,[49] may represent effective strategy for prevention and treatment of cGVHD.

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Authorship

Contribution: H.N. conducted the experiments, analyzed the data, and wrote the manuscript; Y.M. designed the experiments, supervised the research, and wrote the manuscript; H.S., K.K., Y.Y., S.K., and H.U. performed the research; K.T., T. Tanaka, and T.Y. performed histopathologic analyses of the organs; Y.I. provided vital new reagents for the study; and T. Teshima and M.T. supervised the research.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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