て、初回投与薬剤の効果は既報とほぼ同様であった。一方、複数の薬剤が試みられた症例では、先行薬剤の影響とともに薬剤併用効果も考慮する必要があり、各治療薬剤の効果を厳密に判定することはしばしば困難である。従って、本調査における各薬剤の効果は報告者の判断に従った。ただし、再発率や生存率の解析は、寛解維持に用いられた薬剤を各々の症例における key drug として CsA 有効群、PSL 有効群および CY 有効群に分類して行った。

PSL 治療では、その 80%が 24 ヶ月以内に再発することが報告されている(1)。また CsA 登場以前に PSL や PSL/CY 等で治療された特発性後天性 PRCA の生存期間中央値は 14 年、続発性は 4 年であった(27)。本調査において初めて明らかになったことの一つは CsA で治療した場合の再発率と生存率であり、CsA 有効群の再発率は 36%（観察期間中央値 38 ヶ月）、PSL 群の再発率は 57%（同，37 ヶ月）であった。K-M 法による解析で、PSL 群はほぼ 2.5 年以内に全例が再発すると推測されるのに対し、CsA 有効群では 50%無再発期間が約 7 年と推定され、有意差は認められないものの（$p<0.114$）、PSL 有効群より長期の寛解を維持できる可能性が示唆された（図 3）。CsA 有効群で膜性腎症に基づく慢性腎不全による 1 例の死亡があったが、CsA 有効群と PSL 有効群の全生存率に差を認めなかった（図 4）。

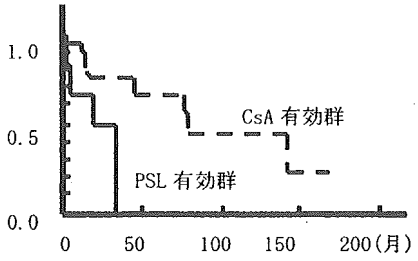

図 3. 無再発生存率

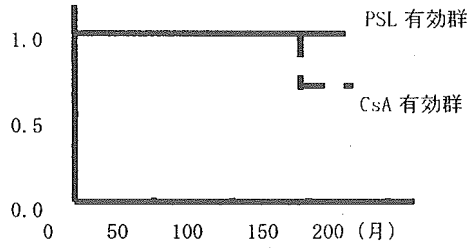

図 4. 全生存率

再発要因として興味深いのは寛解維持療法の有無であった。CsA 有効群における再発 9 例のうち 7 例が維持療法中止後の再発であり、維持療法が施行されていた 16 例での再発は 2 例のみであった。CsA 中止から再発までの期間は初回再発で 9.4±14.2 ヶ月（1.5ー40 ヶ月）、2 回目の再発で 3.5 ヶ月ー10 ヶ月であった。CsA 中止後も寛解を維持している 2 症例（観察期間はそれぞれ 0 ヶ月，1 ヶ月）では観察期間が十分ではなくその評価は現時点で困難である。対照的に、PSL 有効群では全ての再発が維持療法施行中であった。両群間におけるこの解離は維持療法期間の差に依存していなかった。CsA 有効群における維持療法中止例の最長維持療法期間は 78 ヶ月（6.5 年）、また最長維持療法継続例は 168 ヶ月（14 年）であった。対照的に PSL 有効群では全ての再発症例が 31 ヶ月以内の維持療法施行中であった。以上から CsA は PSL に比して有効性において優れているのみならず、維持量減量による再発傾向把握と維持量調整による再発防止の観点からも PSL より有効な薬剤であると推定された。また、今回の調査では CsA 有効群と PSL 有効群の副作用出現頻度に差は認められなかったが、維持量投与中の再発に基づく心理的衝撃、初期治療量へ復帰することによる身体的負担、また再発によって要する輸血は最も高価な治療法の一つであることから鑑み、CsA は患者側要因においても心理的・身体的・経済的に PSL より有効な薬剤であると考えられた。また、PSL 有効群においては再発によって CsA への薬剤変更例が多くなり、再々発の時点では全ての症例に CsA が key drug として使用されていた。PSL 有効例では維持療法中にも再発が多いこと、また、再発後は CsA への切り替えや併用を余儀なくされることから、CsA による治療が現時点で最も単純で効果的であると考えられた。

しかし CsA 無効例もまた存在し、そのような症例に対しては CY, ATG などの薬剤によるサルベージ療法が必要となる。今回の調査における CsA 無効例の解析では、CsA 無効群 4 例の初期投与量は 3.3±1.0mg/kg と有効群の 5.3±0.9mg/kg を下回っており、うち無効例 2 例では 6 週間で投与が中止されていた。今回の調査において CsA の効果は 2 週間以内で 45%，1 ヶ月以内で 60%，3 ヶ月以内で 75%、6 ヶ月以内で 85%の症例に認められた。従って、腎機能障害その他の制約がない症例においては CsA 初期投与量は 6mg/kg、投与期間は 3ー6 ヶ月が適切であると考えられた。さらに CsA の減量には慎重を要することが明らかとなった。24 ヶ月以上の寛解を維持していた症例の CsA 維持量が 2.0±0.6mg/kg であったことは、それ以上の減量が困難であることを示唆している。寛解 24 ヶ月後の維持量を 2.0±0.6mg/kg と仮定し、初期投与量平均値（5.3±0.9mg/kg）を起点として CsA 投与 4 ヶ月後から減量を開始したと単純に仮定した場合、CsA 減量速度は 0.5mg/kg/3 ヶ月であった。従って、個々の症例に応じて減量が

行われるべきであるが、一つの目安としては、寛解導入後のCsA減量は3ヶ月ごとに0.5mg/kgを減量し、維持量が初期量の50%前後となった時点ではさらに慎重な減量を行うことが必要であると考えられた。

今回の調査ではまた、初回寛解を維持することの重要性が示唆された。24ヶ月以上の寛解を維持している症例を対象とした場合、CsA維持量は初回寛解で2.0±0.6mg/kgであるのに対し、再発例では2.8±1.8mg/kg，再々発以後では3.9±0.6mg/kgと漸増する傾向があった。寛解維持量と再、再々寛解導入までの期間に有意差は認められなかったものの初回寛解を維持することの重要性を示唆する所見であると思われる。以上、有効性、寛解維持率、寛解持続期間の観点からPRCAに対する第一選択薬はCsAであると考えられた。

(3) 免疫抑制療法

(3-1) シクロスポリン(cyclosporin A, CsA)

特発性PRCAの第一選択薬剤はCsAである【Ⅳ】。CsAは原則的に再生不良性貧血の治療に準じて投与する。しかし、特発性PRCAは極めて再発しやすい疾患であるため、減量には慎重な姿勢が必要である。ネオーラル（CsA）6mg/kg/日を朝夕、分2で投与し、トラフ値を月に1ー2回測定して150ー250ng/ml程度を目安に適宜調節する。軽度の腎機能障害や高齢者の場合は4ー5mg/kg/日の減量投与を考慮する。輸血が不要となるまでの期間は、3/4の症例では通常4週間以内、残りの1/4の症例では3ヶ月以上を要する。そのためCsAは3ー6ヶ月継続し効果判定を行う。CsAの効果が乏しい場合は薬剤コンプライアンスを確認するとともに、診断時に陰性であってもLGLやTCR再構成の有無について再検することが望ましい。CD4/8比が簡便な指標となり、1以下ではTCR再構成が存在する可能性が高い(25)。血球数が回復傾向にある間は投与を続け、血球数の上昇が頭打ちとなり、3ヶ月以上変化が見られない場合にはCsAの減量を試みる【Ⅳ】。減量の速度に関する一定の基準はない。従って、現在のところは、以下の二つの方法が推奨される。一つは、再生不良性貧血に準じて、血球数の低下がみられない場合に3ヶ月ごとに1mg/kgを減量する。またもう一つの方法はより緩徐な減量で、3ヶ月ごとに0.5mg/kgかもしくは10%減量の減量を行う。このように緩徐に減量を行ってもCsA中止により再発する可能性は否定できない。ほとんどの症例では再発を防ぐために維持量を投与することが必要である。減量の間に維持量を決定する。どちらの方法にしても特に注意すべきは初期投与量の50%程度まで減量した時期であり、この時期貧血の再燃をみることが多いため以後の減量は特に注意して行うことが望ましい【Ⅳ】。3ー6ヶ月で寛解が得られない場合は中止して他の治療法を考慮する。CsAの副作用として最も重要なものは腎障害であり予防のためのモニタリングが必要である。その他の副作用は、血圧上昇、多毛、歯肉腫脹、指の震えなどである。

(3-2) プレドニゾロン（prednisolone, PSL)

PSL経口を1mg/kg/日から開始し、寛解が得られるまで継続する。メチルプレドニゾロン（methyprednisolone, mPSL）のパルス療法から開始する場合もある。およそ40%の患者では4週間以内に反応するため、3ヶ月に渡って初期量を投与することは勧められていない。PSLはヘマトクリットが35%に達したらゆっくり減量し、少なくとも3ー4ヶ月をかけて中止するようにする。再発を防ぐために維持量を投与することが必要な場合が多い。減量の間に維持量を決定する【Ⅳ】。初期治療後、80%が24ヶ月以内に再発するとの報告がある(1)。2ー3ヶ月以内に反応が認められない場合はPSLを20ー30mg/日まで急速に減量し、CsAやCYの併用を考慮する、単独あるいはそれぞれの副作用軽減のためにステロイドとの併用が行われる。ステロイドと他の薬剤の併用が耐用性と効果を増強する。CsAとの併用が最も単純で効果的である。

(3-3) シクロホスファミド(cyclophosphamide, CY)

CYを経口で50mg/日から開始し、毎週もしくは2週間ごとに増量し最大150mg/日を維持し、寛解を得るか骨髄抑制がおこるまで続ける。骨髄抑制（白血球数＜2,000/μl または血小板数<10万/μl）が生じた場合は中止し回復を待つ。効果発現まで平均で11ー12週を要する(1)【Ⅳ】。副作用として二次性白血病や二次発癌のほか、白血球減少や免疫抑制による感染を合併することが多く注意が必要である。cholinesterase値は白血球減少の予知因子として報告されており、正常値の65%以下となった場合には注意が必要である（29)【Ⅲ】。3ヶ月以上投与しても効果がない場合、さらに増量する方法もあるが、

CsA が使用可能な今日では非実際的である。

3) 続発性 PRCA の治療

(1) LDGL (lymphoproliferative disease of granular lymphocytes)

ネオーラル (CsA) 6mg/kg/日を朝夕、分2で投与し、トラフ値を月に1ー2回測定して 150ー250ng/ml 程度を目安に適宜調節する。軽度の腎機能障害や高齢者の場合は 4ー5mg/kg/日の減量投与を考慮する。CsA は 3ー6ヶ月継続し効果判定を行う【IV】。反応が得られた後の CsA 減量は特発性 PRCA に準ずる。LDGL に続発する PRCA において CsA の有効率は 80%と、PSL 単独または CY との併用療法の 50-60%に比べて良好である(7,23)。LGL に対し、CY とステロイド併用によって寛解が得られた後の再発は少ないとの報告がある(6)。限られた症例における後方視的な検討より得られたものであるが、CsA 抵抗例には CY または PSL などの単独、またはメソトレキセート (methotrexate, MTX) を試みる(7,23)。

(2) 胸腺腫

胸腺腫があれば摘出を考慮する。有効率は 30%ー40%で、4ー8 週で効果が現れる。胸腺摘出が困難な症例や摘出に反応しない場合は特発性 PRCA として治療を行う(1) 【IV】。

(3) 骨髄異形成症候群(MDS)

染色体異常を有する PRCA では免疫抑制療法が無効であるとの報告が多い(1,2)。ただし、CsA の有効性を示唆する報告もあり(30)試みる余地がある。

(4) リウマチ性疾患

リウマチ性疾患に続発する PRCA は PSL の維持量投与中に発症する場合がある。原疾患の病態に応じてメチルプレドニゾロン (methylprednisolon, mPSL) パルス療法を選択する場合もあるが、無効の場合には CsA を用いるべきである【IV】。

16. 難治例・再発例への対応

CsA が無効の時、投与量と投与期間が適正であったかを検証し、さらに続発性の可能性、特に LDGL の除外やヒトパルボウイルスの持続感染の有無を確認する。また、再発例に対しては CsA や PSL の減量・中止の速度が適正であったか否かを確認する。再発例の多くは CsA に反応するので、この場合も CsA が第一選択となる(28) 【IV】。CsA 無効例に対しては、抗胸腺グロブリンもしくはステロイドと CY の併用が有効な場合がある(1,7)。難治例には mPSL 大量療法や大量ガンマグロブリン療法、抗胸腺グロブリン療法や抗リンパ球グロブリン療法、血漿交換療法などが試みられている(31)。近年、難治例や CLL に伴う PRCA に対する抗 CD20 抗体(32,33)や、他の治療で難治性であった CLL および LGL 白血病に伴う PRCA に対して抗 CD52 抗体 (Campath-1H) を試み、有効であったことが報告された(14)。また血縁者間同種造血幹細胞移植の成功例も報告されている(34)。また、摘出が不能で、化学療法抵抗性の胸腺腫に伴った PRCA にオクトレオチド (octreotide) と PSL の併用投与が試みられ、胸腺腫の消退と PRCA の完全寛解が得られている (35)。厳密な試験は行われていないが摘脾の有効率は 17%と報告されており(1), すべての免疫抑制療法に抵抗性で再発を繰り返す若年者には最終的な手段の一つかも知れない。LDGL においては CsA も有効であるが(80%)(7)、不能例においては CY 単独またはステロイドとの併用が有効であり (52%)、長期の寛解維持が期待されることが示唆されている(5)。

17. 治療管理に関わる事項について

輸血は時に長期に渡るため白血球除去赤血球を用いる。頻回輸血例では鉄過剰症として、肝障害、糖尿病、性腺機能低下・内分泌障害、皮膚色素沈着、心不全、関節症状、易感染性が出現するようになる。治療には鉄キレート剤としてデスフェリオキサミン(desferrioxamine) の静脈内投与を行う。PSL、CsA、CY 使用時は易感染性であるので注意を要する。また、各薬剤特有の副作用に注意が必要である。

18. 中・長期の経過

特発性 PRCA のおよそ 5ー10%は自然緩解する(1)。PSL 治療後の再発は 2 年間で 80%であるが CsA や CY が初回治療に用いられた場合の再発率はこれまで不明であった。CsA 治療例において再発要因として最

も重要なものは寛解維持療法の有無である。CsA中止から再発までの期間は初回再発で9.4±14.2ヶ月、2回目の再発で3.5ヶ月ー10ヶ月であった。対照的に、PSL有効群では全ての再発が維持療法施行中であり 寛解から31ヶ月以内の再発であった。またCsA維持量は再発例では2.8±1.8mg/kg, 再々発以後では3.9±0.6mg/kgと漸増する傾向があり、初回寛解維持の重要性が示唆される。CsAやPSL投与による再発後においても60%の患者が長期の経過の中で輸血不要となる(1)。特発性後天性PRCAの平均生存期間は1984年における37症例の解析で14年と報告されている。PRCAから再生不良性貧血への移行は稀ではあるが報告されており、GLPDの関与が推定されている(36)。一方で、難治性PRCAの3ー5%が白血病を発症したとの報告がある。MDSもしくは前白血病状態であった可能性も否定できない。

19. 予後予測因子

原発性PRCAのうち、特発性PRCAの予後予測因子として、CFU-E数が正常の場合は免疫抑制療法に対する反応性が良好であるとの報告がある(1)。染色体異常を呈するPRCAは治療反応性に乏しく予後不良である。特発性PRCAに較べて続発性の生存期間中央値は4年と短く(27)、病型分類のための鑑別診断が重要である。難治例ではヘモクロマトーシスによる臓器障害によって予後が規定される。

20. 今後に残された問題点と将来展望

PRCA の治療においては再発が多く次第に治療抵抗性となるため、どのように寛解を得るかということより、どのように寛解を維持するかが重要であるとの観点から、欧米では初回寛解効果はCsAに劣るもののPSLが第一選択の薬剤であるとされてきた。今回の「赤芽球癆診療の参照ガイドの作成を目指したアンケート調査」(28)によって、有効性、寛解維持率、寛解持続期間の観点からPRCAに対する第一選択薬はCsAであることが明らかとなった。しかし、CsAがPRCAの治癒をもたらすという可能性は現時点で乏しい。従って、寛解を維持するためにはCsAの維持量投与が不可欠である。今後の問題点は、CsAをkey drugとしつつ、いかにCsAからの離脱を可能にするかという点にある。本調査においてもCsAからの離脱を目指して抗胸腺グロブリン (anti-thymocyte globulin, ATG) の投与が2例において試みられていたがCsAの中止は不可能であった。CYによって寛解が得られたPRCA では維持療法中止後も長期の寛解が持続するとの報告があるが、寛解導入有効率の点からCYはCsAに及ばない。またCYの副作用を鑑みれば、比較的少量のCsAで寛解維持が可能な症例に対し、離脱を目指してCY投与を試みることには躊躇を伴う。近年、難治例やCLLに伴うPRCAに対する抗CD20抗体(33,34)や、他の治療で難治性であったCLLおよびLGL白血病に伴うPRCAに対して抗CD52抗体 (Campath-1H) (24)を試み、有効であったことが報告されている。これらの新規治療薬を用いることでPRCAの治癒をもたらすことが可能か否かについて今後検証が必要である。さらに、PRCA の治癒を目指すためには、その本態の解明に向けた基礎的研究もまた必要不可欠であり、特に造血幹細胞を視点に据えた自己免疫機序の解明が望まれる。

21. 問題点の解決のために現実に進められている研究や必要な取り組み

現在、LDGL の治療成績を前方視的に解析するため、CALGB (Cancer and Leukemia Group B) でCsAをフロントラインとした臨床試験、ECOG (Eastern Cooperative Oncology Group) でMTXとCYのクロスオーバー試験が進行中である(37)。続発性PRCAの治療については今回行った「赤芽球癆診療の参照ガイドの作成を目指したアンケート調査」をもとに解析が進行中である。

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Infliximab Induces Potent Anti-inflammatory Responses by Outside-to-Inside Signals Through Transmembrane TNF-α

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Background & Aims: Both infliximab (chimeric anti-tumor necrosis factor [TNF]-α antibody) and etanercept (p75 TNF-α receptor/immunoglobulin G fusion protein) are effective against rheumatoid arthritis, but only infliximab induces clinical remission in Crohn's disease. To clarify this difference in clinical efficacy, we investigated reverse signaling through transmembrane TNF-α (mTNF) by these 2 anti-TNF agents. *Methods:* We stably transfected wild-type and cytoplasmic serine-replaced mutant forms of mTNF in human Jurkat T cells. Cells were stimulated with infliximab and etanercept and then analyzed for E-selectin expression, reactive oxygen species accumulation, apoptosis, and cell cycle distribution by flow cytometry. Interleukin-10 and interferon-γ were measured by enzyme-linked immunosorbent assay. Phospho-c-Jun NH2-terminal kinase, Bax, Bak, $p21^{WAF1/CIP1}$, caspase-8, and caspase-3 were examined by immunoblotting. *Results:* Both anti-TNF agents induced E-selectin expression, but only infliximab induced interleukin-10 production, apoptosis, and G0/G1 cell cycle arrest. Apoptosis and cell cycle arrest were abolished by substitution of all 3 cytoplasmic serine residues of mTNF by alanine residues. Infliximab induced accumulation of reactive oxygen species and up-regulation of Bax, Bak, and $p21^{WAF1/CIP1}$ proteins, suggesting the involvement of p53 activation. Moreover, phosphorylation of c-Jun NH2-terminal kinase was necessary for infliximab-induced apoptosis and cell cycle arrest. *Conclusions:* We revealed the mTNF motifs and the downstream intracellular molecular events essential for reverse signaling through mTNF. The biologic effects of mTNF elicited by infliximab should be important action mechanisms of this potent anti-inflammatory agent in addition to the neutralization of soluble TNF-α. These observations will provide insight into the novel role of mTNF in inflammation.

Tumor necrosis factor (TNF)-α is a potent proinflammatory cytokine that orchestrates various inflammatory responses. The precursor form of TNF-α, called *transmembrane TNF-α* (mTNF), is expressed as a 26-kilodalton cell surface type II polypeptide on activated macrophages and lymphocytes, as well as on other cell types. mTNF consists of N-terminal 30 amino acid (aa) residues of the cytoplasmic domain, 26 aa of the transmembrane domain, and 177 aa of the extracellular domain. The C-terminal 157 aa are processed by TNF-α-converting enzyme (TACE) between residues Ala^{76} and Val^{77}.[1,2] The secreted soluble form of 17-kilodalton polypeptide binds to type 1 and type 2 TNF receptors (TNF-RI, TNF-RII) as a homotrimer and mediates pleiotropic effects, such as cytokine production, cell adhesion molecule expression, and proliferation as well as apoptosis.[3–5]

mTNF is constitutively expressed on resting natural killer (NK) cells[6] and is also induced upon activation on various types of cells such as monocytes and T lymphocytes.[7,8] mTNF has also been shown to mediate biologic functions in a cell-to-cell contact fashion and to be involved in cytotoxic activity by monocytes[7,9] and NK cells,[6] polyclonal B-cell activation induced by human immunodeficiency virus (HIV)-infected $CD4^+$ T cells[10] and by human T-cell leukemia virus-infected $CD4^+$ T cells,[11] IL-10 production from monocytes,[8] and expression of adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and tissue factor from endothelial cells.[12,13] Costimulatory signals for interleukin (IL)-4-dependent immunoglobulin synthesis are also provided by mTNF.[14]

mTNF not only acts as a ligand but also mediates reverse signaling into cells expressing this molecule. Our group and others have shown that stimulation of mTNF with anti-human TNF-α polyclonal Ab or TNF-RII

Abbreviations used in this paper: aa, amino acid; CHX, cycloheximide; Eta, etanercept; Ifx, infliximab; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; mTNF, transmembrane TNF-α; ROS, reactive oxygen species; Ser, serine; TACE, TNF-α converting enzyme; TNF, tumor necrosis factor; TNF-R, TNF receptor; WT, wild-type.

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expressed on HeLa cells induces several biologic effects, including calcium mobilization,[11,15] cytokine production,[11] and E-selectin expression in T cells.[16] The cytoplasmic domain of mTNF should play a critical role in reverse signaling, considering its extreme aa conservation (nearly 90%) among different animal species.[17] Phosphorylation of human mTNF by anti-TNF-α polyclonal Ab in some monocytic cells and in 26-kilodalton precursor TNF-α-transfected HeLa cells has been reported and shown to be restricted to serine (Ser) residues.[18]

The beneficial effect of anti-TNF therapy has been recognized by large-scale, long-term studies of rheumatoid arthritis[19,20] and Crohn's disease.[21] In smaller study-scale studies, the efficacy of anti-TNF therapy has also been demonstrated in patients with psoriasis,[22] ankylosing spondylitis,[23,24] and Behçet's disease.[25] These successful clinical trials have clarified the importance of TNF-α in the pathogenesis of chronic inflammatory disorders. There are 2 types of anti-TNF-α agent: one is an antibody against human TNF-α (infliximab; chimeric anti-TNF-α monoclonal antibody [mAb] with murine variable regions and human IgG1 constant regions),[26] and the other is made of a soluble form of human TNF-RII fusion protein (etanercept; recombinant TNF-RII/IgG1 Fc domain fusion protein).[27] Both of these agents have been shown to bind to soluble TNF and mTNF[26–28] and to be equally effective in rheumatoid arthritis. However, in Crohn's disease, remission is induced only by infliximab and not by etanercept.[29] This difference between infliximab and etanercept in a clinical setting might indicate the existence of additional biologic functions of anti-TNF agents other than mere neutralization of TNF-α.

Here, we demonstrate that outside-to-inside (reverse) signals through mTNF elicited by infliximab resulted in increased apoptosis and cell cycle arrest in a human T-cell line. Moreover, we identified the mTNF intracellular motifs, as well as the downstream intracellular molecules essential for reverse signaling.

Materials and Methods

Cell Line and Reagents

Jurkat cells, a human lymphoblastoid T-cell line, were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL streptomycin at 37°C in a 5% CO_2-humidified atmosphere. Infliximab (Remicade) was provided by Tanabe Seiyaku Co. Ltd. (Osaka, Japan), and etanercept (Enbrel) was provided by Wyeth (Tokyo, Japan). Rituximab (chimeric anti-human CD20 mAb), which was used as a control chimeric Ab, was purchased from Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). Cycloheximide (CHX) was obtained from Sigma (St. Louis, MO); 2′,7′-dichlorofluorescein diacetate was supplied by Wako Chemical (Osaka, Japan); F(ab′)2 fragment of rabbit anti-human IgG was supplied by Dako Cytomation (Kyoto, Japan); [^{3}H] thymidine was obtained from Amersham Biosciences (Arlington Heights, IL); SB 203580 and PD98059 came from Promega (Madison, WI); and SP600125 and z-VAD-fmk were obtained from Calbiochem (La Jolla, CA).

Mutagenesis of mTNF

Mutant mTNF resistant to TACE-mediated cleavage was generated by the oligodeoxyribonucleotide-directed amber method (site-directed mutagenesis) as described previously.[16] In this uncleavable form of mTNF designated as wild-type (WT), Arg at aa residue 77 and Ser at aa residue 78 of the native mTNF were both replaced by Thr (R77T/S78T), which has already been shown to result in the effective reduction of the cleavage of mTNF.[18] The cytoplasmic Ser at aa residues 2, 5, and 27 was sequentially replaced by Ala by site-directed mutagenesis in accordance with the manufacturer's instructions (Mutan-Super Express Km kit; Takara Shuzo, Kyoto, Japan). Briefly, mutations were introduced into the uncleavable form of mTNF (R77T/S78T) subcloned into pKF19 by the following oligonucleotides: TCGGCTTATGGCCACTGAAAGCATGA for S2A, ATGAGCACTGAAGCCATGATCCGG for S5A, and GCCCCAGGGCGCCAGGCGGTG for S27A. The nucleotide sequences for the mutant forms of mTNF were confirmed by direct sequencing using an Amplicycle sequencing kit (Perkin-Elmer, Norwalk, CT).

Stable Expression of mTNF on Jurkat Cells

WT and cytoplasmic Ser-replaced mutant mTNFs were cloned into pCXN2 mammalian expression vector (kindly provided by Dr. Jun-ichi Miyazaki, Osaka University) and transfected into Jurkat cells by electroporation using Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, CA) at 240 V, 960 μF. The cells were immediately plated on prewarmed medium and cultured at 37°C. Two days after transfection, the cells were selected in the presence of G418 (Sigma) 1.6 mg/mL. Clones of each mutant form of mTNF were obtained by limiting dilution.

Surface Staining

Cells were washed twice with the staining medium PBS containing 2% FBS. Cells (5×10^5 per sample) were stained on ice for 40 minutes with FITC-conjugated mAbs in staining medium. Expression of mTNF, TNF-RI, TNF-RII, and Fas was studied using anti-human TNF-α mAb (R&D Systems, Minneapolis, MN), anti-human TNF-RI mAb (Genzyme, Cambridge, MA), anti-human TNF-RII mAb (Genzyme), and anti-human Fas mAb (Medical and Biological Laboratories [MBL], Nagoya, Japan). Induction of E-selectin was studied by using anti-human E-selectin (CD62E) mAb (Ancell, Bayport, MN). FITC-conjugated mouse IgG1 (Dako) was used as a negative control. Stained cells were washed twice with staining medium, and then the expression of cell surface

molecules was measured using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using CELLQuest software (Becton Dickinson).

Binding Assay of Infliximab and Etanercept to mTNF

mTNF-transfected Jurkat cells were incubated with infliximab, etanercept, or a control chimeric antibody, rituximab (all at 10 μg/mL) for 30 minutes at 4°C, followed by staining with an FITC-conjugated rabbit anti-human F(ab')2 as a secondary antibody for FACS analysis.

cDNA Expression Array

WT mTNF-transfected Jurkat cells were stimulated for 6 hours with rabbit anti-human TNF-α polyclonal Ab (Genzyme) or normal rabbit IgG (Genzyme) at 10 μg/mL. Next, expression of mRNA was analyzed using Atlas cDNA expression array (Clontech, Palo Alto, CA), in accordance with the manufacturer's protocol.

Cytokine ELISA

mTNF-transfected Jurkat cells (1×10^6 cells/mL) were incubated for 24 hours with infliximab, etanercept, or rituximab 10 μg/mL in 24-well plates. Culture supernatants were collected, and concentrations of IL-10 and interferon (IFN)-γ in each culture supernatant were determined by using commercially available ELISA kits, in accordance with the recommendations of the manufacturer (Biosource, Camarillo, CA). In addition, soluble TNF levels in 48-hour culture supernatants of mTNF-transfected Jurkat cells (1×10^6 cells/mL) were measured by ELISA kits (R&D Systems).

Proliferation Assays

WT mTNF-transfected Jurkat cells were cultured in triplicate wells of 96-well flat-bottomed plates (5×10^4 cells/well) in RPMI1640 supplemented with 10% FBS. Cells were stimulated with infliximab, etanercept, or rituximab at 1 or 10 μg/mL for the indicated time periods. During the last 9 hours of culture, cells were pulsed with 1 μCi [^{3}H] thymidine per well. Incorporated radioactivity was measured with a β-scintillation counter. Cross-linking of etanercept was performed as follows. WT mTNF-transfected Jurkat cells were stimulated for the indicated time periods with etanercept at 10 μg/mL with or without 10 μg/mL of rabbit anti-human IgG F(ab')2.

Apoptosis Assay

WT and Ser-replaced mutant mTNF-transfected Jurkat cells were stimulated for 24 hours with infliximab, etanercept, or rituximab 10 μg/mL. Apoptosis and cell death were measured with a MEBCYTO Apoptosis Kit (MBL), as described previously.[30] To see the effect of de novo protein synthesis and the involvement of the MAPK pathway in apoptosis signaling, different concentrations of CHX, SB 203580, PD98059, or SP600125 were administered to the Jurkat cells for 30 minutes before treatment with infliximab 10 μg/mL, followed by Annexin V/PI staining. Cross-linking of etanercept was performed as follows. WT mTNF-transfected Jurkat cells were stimulated for 24 hours with etanercept 10 μg/mL with or without rabbit anti-human IgG F(ab')2 10 μg/mL, followed by flow cytometry for Annexin V/PI-positive cells.

Cell Cycle Analysis

The mTNF-transfected Jurkat cells were stimulated with 10 μg/mL infliximab, etanercept, or rituximab for the indicated time periods. The treated cells were washed twice with PBS and fixed for 60 minutes with ice-cold 70% ethanol. DNA was stained for 30 minutes with propidium iodide (PI; Sigma) 10 μg/mL, containing RNase (Sigma) 1 mg/mL. Samples were analyzed on a flow cytometer (Becton Dickinson). G0/G1, S, G2/M, and Sub-G1 values were determined by ModFit LT analysis software (Verify Software House, Topsham, ME). To see the involvement of caspases, WT mTNF-transfected Jurkat cells were untreated or pretreated with 50 μmol/L of z-VAD-fmk for 30 minutes, followed by stimulation for 48 hours with infliximab at 10 μg/mL and analysis for PI staining.

Western Blot Analysis

WT mTNF-transfected Jurkat cells were incubated in 6-well plates with infliximab, etanercept, or rituximab at 10 μg/mL. Then cells were lysed in SDS sample buffer (0.625 mol/L Tris-HCl [pH 8.8], 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 10% β-mercaptoethanol), and the lysates were boiled for 5 minutes and then analyzed by SDS-PAGE. They were then transferred to nitrocellulose membrane. The membranes were incubated for 1 hour in Tris-buffered saline (TBS) containing 5% nonfat milk. After incubation, the membranes were blotted overnight using rabbit anti-caspase-8 polyclonal Ab (BD Pharmingen, San Diego, CA), rabbit anti-cleaved caspase-3 (ASP175) polyclonal Ab, rabbit anti-Bax polyclonal Ab, rabbit anti-Bak polyclonal Ab, rabbit anti-$p27^{KIP1}$ polyclonal Ab, mouse anti-$p21^{WAF1/CIP1}$ (DCS60) mAb, rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) polyclonal Ab (Cell Signaling Technology, Beverly, MA), or mouse anti-β-actin mAb (Sigma). After being washed with TBS containing 0.5% Tween 20 (TBS-T), the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG Ab or goat anti-rabbit IgG Ab (Southern Biotechnology, Birmingham, AL) for 1 hour and visualized with ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech, Piscataway, NJ). To see the involvement of the JNK pathway in infliximab signaling, different concentrations of SP600125 were administered to the Jurkat cells 30 minutes before treatment with infliximab at 10 μg/mL.

Measurement of Reactive Oxygen Species

WT mTNF-transfected Jurkat cells were stimulated with infliximab, etanercept, or rituximab for 6, 12, 18, or 24 hours. Next, the stimulated cells were labeled with a cell-permeable fluorescent dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA). When DCFH-DA is oxidized by reactive

oxygen species (ROS) in the cells, the fluorescent signal increases and is detectable by flow cytometry.

Statistical Analysis

Results were expressed as means ± SD. Significance was determined by an unpaired 2-tailed Student *t* test.

Results

Expression of Uncleavable Form of mTNF and Cytoplasmic Ser Residue-Replaced mTNF Mutants on Jurkat Cells

TACE-resistant mTNF has been generated as described previously,[16] which was designated here as WT mTNF. Here, we constructed mTNF mutants lacking cytoplasmic Ser residues. The cytoplasmic domain of mTNF has 3 Ser residues at aa positions 2, 5, and 27. These Ser residues were sequentially replaced by Ala residues by site-directed mutagenesis. The structures of WT, S2A, S5A, S27A, S5A/S27A, S2A/S27A, S2A/S5A, and S2A/S5A/S27A are shown schematically in Figure 1*A*. The expression levels of mutant forms of mTNF were almost the same, as assessed by fluorescence intensity using FITC-conjugated anti-human TNF-α mAb (Figure 1*B*). Wild-type and mock-transfected Jurkat cells did not express detectable levels of cell surface mTNF by flow cytometry. We previously indicated that both infliximab and etanercept bound to wild-type cleavable mTNF[28] and here examined the binding activity of infliximab and etanercept to these Ser mutant forms of uncleavable mTNF. Both infliximab and etanercept bound clearly to S2A/S5A/S27A of mTNF (Figure 1*C*) and the other mutant forms of mTNF (data not shown). We also confirmed the lack of expression of TNF-RI and TNF-RII on the Jurkat cells, using a flow cytometer (data not shown).

Induction of E-Selectin on Jurkat Cells by Stimulating mTNF With Infliximab and Etanercept

Our group has already reported that infliximab and etanercept bind equally to mTNF on Jurkat cells.[28] We next examined whether these drugs could transmit outside-to-inside signals through mTNF. After stimulation of mTNF for 6 hours with infliximab, etanercept, or rituximab at 1 μg/mL, we studied the expression of E-selectin by flow cytometry. Rituximab is a chimeric anti-human CD20 (B-cell-specific) mAb and was used here as a control Ab. E-selectin was induced on WT mTNF-transfected Jurkat cells by infliximab and etanercept but not by rituximab (Figure 2*A*). Neither infliximab nor etanercept induced E-selectin on wild-type Jurkat cells that were not carrying mTNF on their surfaces (data not shown). We next investigated the induction of E-selectin on Jurkat cells transfected with Ser-replaced mTNF mutants. The levels of E-selectin expression upon activation with infliximab and etanercept were almost the same between Jurkat cells transfected with either of the mTNF mutants and those transfected with WT mTNF. The data of S2A/S5A/S27A are shown as a representative in Figure 2*B*.

IL-10 Production Is Induced by Infliximab but not by Etanercept

We studied the effect of these anti-TNF agents on cytokine expression because various cytokines are involved in the development of chronic inflammatory diseases. By stimulating mTNF with rabbit anti-human TNF-α polyclonal Ab for 6 hours, IL-10 mRNA was 2.6-fold up-regulated on cDNA expression array analysis (data not shown). WT mTNF-transfected Jurkat cells were incubated for 24 hours with infliximab, etanercept, or rituximab at 10 μg/mL, and the expression of IL-10 and IFN-γ in the culture supernatants was measured by ELISA (Figure 3). As expected from the cDNA expression array analysis, infliximab induced a 2.7-fold increase in IL-10 production with statistical significance ($P < .05$), whereas stimulation with etanercept did not induce IL-10 production. Production of IFN-γ was not significantly different among these agents. To assess the involvement of cytoplasmic Ser residues, we investigated IL-10 production in Ser-replaced mTNF mutants. Infliximab-induced IL-10 production tended to be reduced in Ser-replaced mutants; however, there was no significant difference. The data of S2A/S5A/S27A are shown as a representative in Figure 3*A*. IFN-γ production was unchanged in S2A/S5A/S27A (Figure 3*B*). The concentrations of soluble TNF of WT and S2A/S5A/S27A were 1304 ± 241 pg/mL and 853 ± 56 pg/mL (mean ± SD), respectively.

Cell Proliferation Is Suppressed by Stimulation of mTNF With Infliximab but not With Etanercept Alone

We next studied the effects of infliximab and etanercept on cell proliferation. Cells were stimulated for 72 hours with infliximab, etanercept, or rituximab at 1 or 10 μg/mL (Figure 4*A* and 4*B*). Proliferation of WT mTNF-transfected Jurkat cells was almost completely abolished after 24 hours of infliximab treatment. In contrast, cell proliferation was barely suppressed by etanercept. The magnitude of inhibition of cell prolifera-

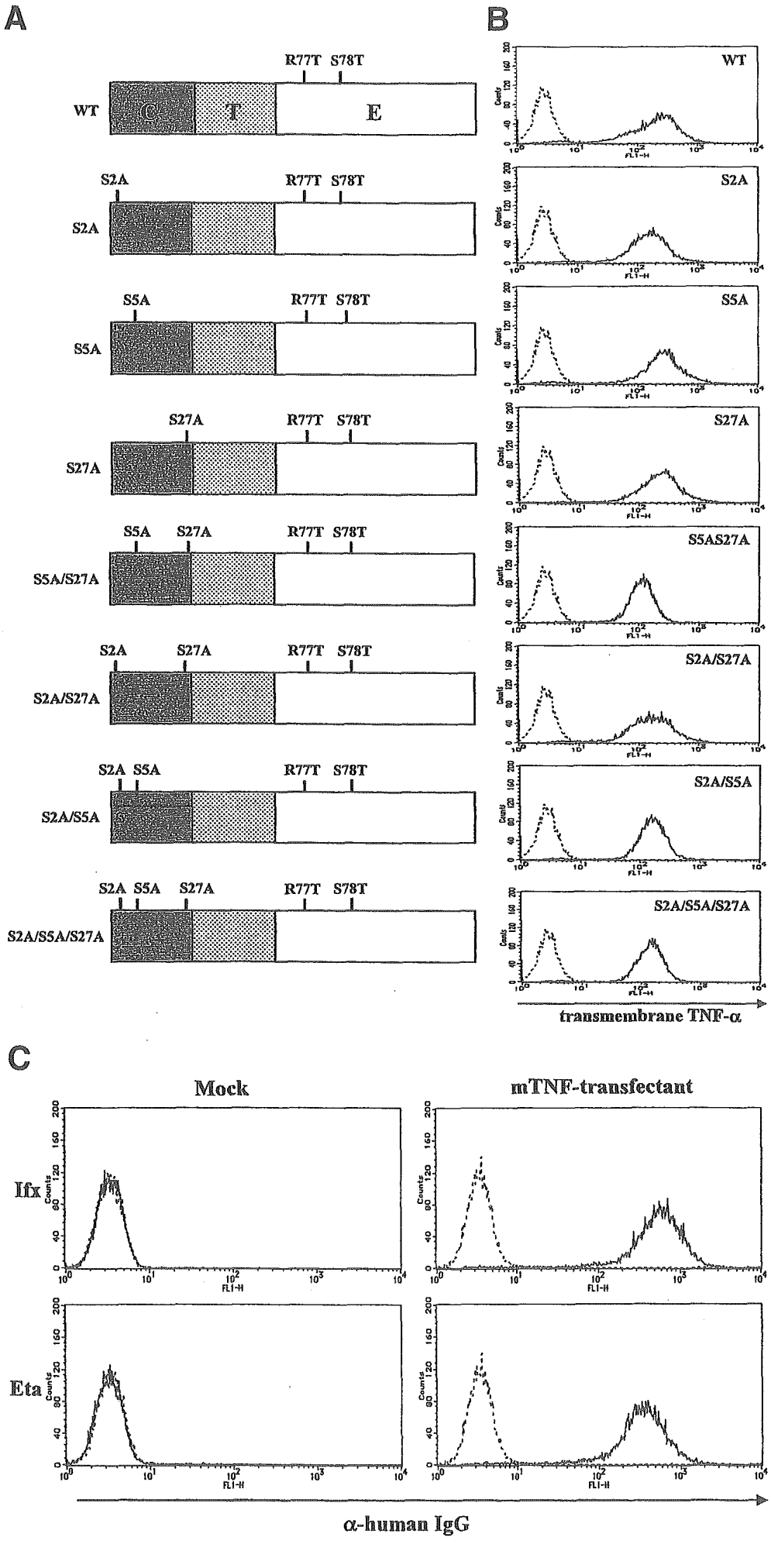


Figure 1. Uncleavable form of mTNF (WT mTNF) and cytoplasmic Ser-replaced mutant forms of mTNF are stably expressed on Jurkat cells. (*A*) Structures of WT and mutant forms of mTNF. C, cytoplasmic domain; T, transmembrane domain; E, extracellular domain. mTNF cDNA was obtained from normal human PBMCs by RT-PCR of total RNA. Arg^{77} and Ser^{78}, just upstream from the cleavage site (Ala^{76}-Val^{77}) of TNF-α converting enzyme, were replaced by Thr residues (R77T/S78T) by site-directed mutagenesis (designated as WT). Moreover, cytoplasmic Ser residues at aa positions of 2, 5, and 27 of WT mTNF were sequentially substituted by site-directed mutagenesis. Substitutions of 1 Ser residue are designated as S2A, S5A, and S27A, respectively. Substitutions of 2 Ser residues are S5A/S27A, S2A/S27A, and S2A/S5A. Substitutions of all cytoplasmic Ser residues are denoted by S2A/S5A/S27A. All mutants were subcloned into pCXN2 expression vector and were stably expressed in Jurkat cells. (*B*) Cell surface expression levels of mTNF on transfected Jurkat cells. Cells were stained with FITC-conjugated mouse anti-human TNF-α mAb (*solid lines*) and analyzed by flow cytometry. Background staining is shown by *dotted lines* (mouse IgG1/FITC). (*C*) Binding activity of infliximab and etanercept to transmembrane TNF-α. Mock-transfected and mTNF-transfected Jurkat cells were incubated with infliximab (Ifx), etanercept (Eta), or control Ab, rituximab. Cells were then stained with an FITC-conjugated goat anti-human IgG F(ab')2 as a secondary antibody for FACS analysis. The histograms show the binding of infliximab, etanercept (*solid line*), and control Ab (*dotted line*) to mock-transfected or mTNF-transfected Jurkat cells (S2A/S5A/S27A is indicated as a representative).

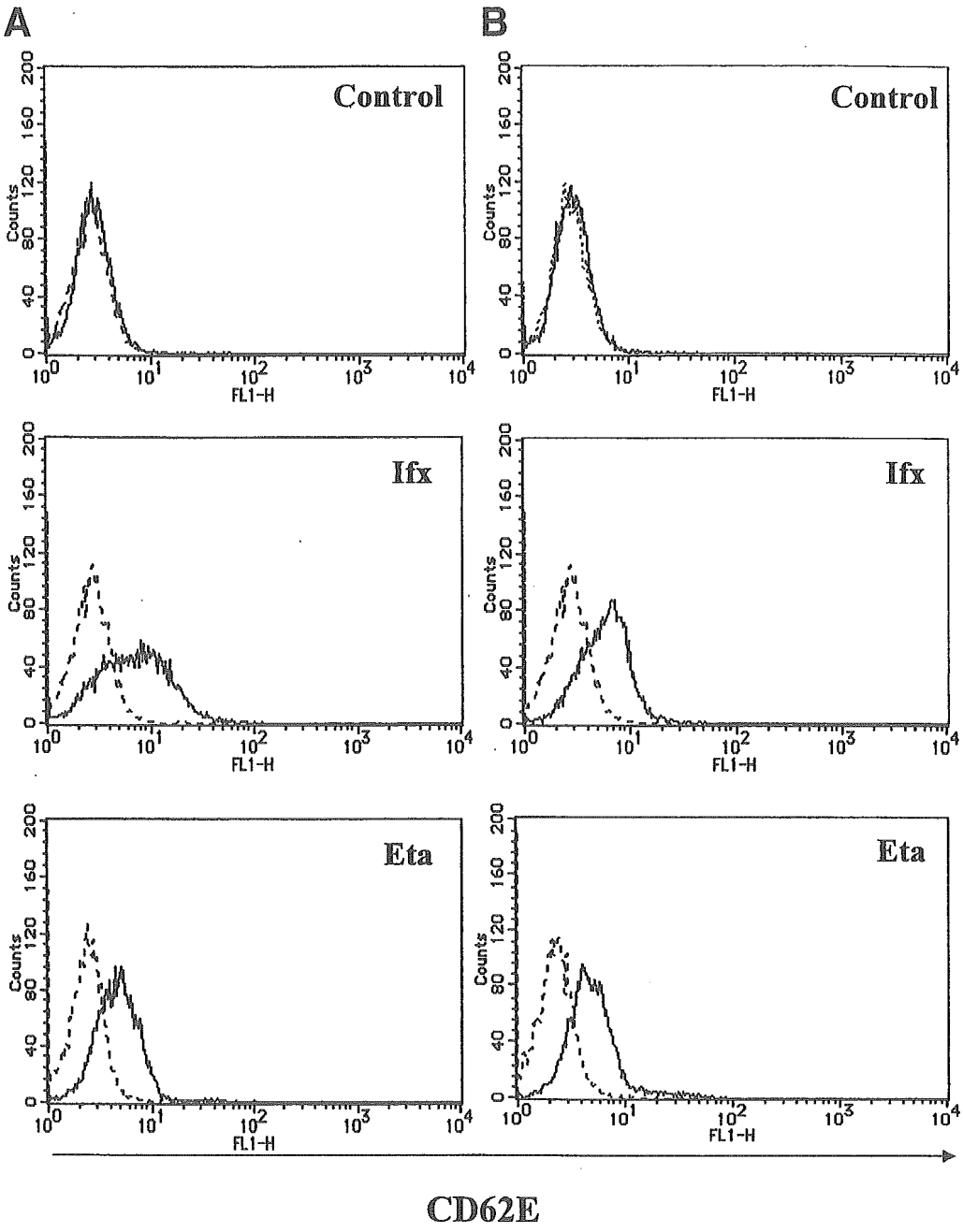


Figure 2. E-selectin is induced on mTNF-transfected Jurkat cells with stimulation of mTNF by both infliximab and etanercept. WT (*A*) and S2A/S5A/S27A (*B*) mTNF-transfected Jurkat cells were stimulated for 6 hours with infliximab (Ifx), etanercept (Eta), or a control Ab, rituximab, at 1 μg/mL. Cells were stained with FITC-conjugated anti-CD62E (E-selectin) mAb (*solid lines*) and analyzed by flow cytometry. Background staining is shown by *dotted lines*.

tion was clearly different between infliximab and etanercept.

We then analyzed the effect of cross-linking of etanercept on cell proliferation. Cross-linking of cell surface etanercept with anti-human IgG induced significant suppression of cell proliferation, but its effect was less pronounced compared with that of infliximab (Figure 4*C*).

Apoptosis of mTNF-Transfected Jurkat Cells Is Dramatically Induced by Infliximab but not by Etanercept Alone

To clarify the mechanisms by which infliximab inhibited cell proliferation, we first investigated apoptosis induction. WT mTNF-transfected Jurkat cells were stimulated for 24 hours with infliximab, etanercept, or rituximab at 10 μg/mL. Stimulation with infliximab dramatically increased the number of Annexin V-positive cells from 5.1% to 68.9%, whereas stimulation with etanercept had no effect on apoptosis (Figure 5*A*). According to the kinetic assay, numbers of Annexin V-positive cells began to increase 12 hours after the initiation of incubation with infliximab (data not shown).

We next examined the infliximab-induced apoptosis of Jurkat cells transfected with Ser-replaced mTNF mutants. The apoptotic effect of infliximab was completely abolished in the S2A/S5A/S27A mutant (Figure 5*B*). We

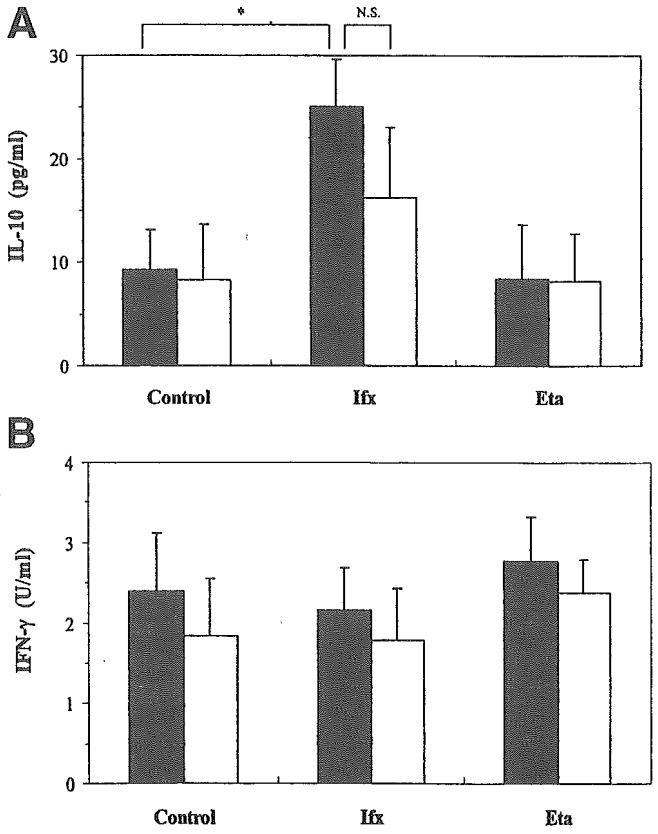


Figure 3. IL-10 is produced from mTNF-transfected Jurkat cells by stimulation with infliximab but not with etanercept. WT (*solid bars*) and S2A/S5A/S27A (*open bars*) mTNF-transfected Jurkat cells were cultured for 24 hours with infliximab (Ifx), etanercept (Eta), or a control Ab, rituximab, at 10 μg/mL. Concentrations of IL-10 (*A*) and INF-γ (*B*) in the culture supernatants were measured by ELISA. The *columns* and *bars* represent the mean and SD of triplicate cultures. Statistical evaluation was conducted by paired Student *t* test (*$P < .05$; NS, no significance).

also studied apoptosis induced by infliximab in Jurkat cells transfected with the other Ser-replaced mutants (Figure 5*C*). S2A and S5A showed considerable degrees of inhibition of apoptosis, whereas S27A alone had no effect on infliximab-induced apoptosis. Substitution of 2 Ser residues (S5A/S27A, S2A/S27A, and S2A/S5A) had more inhibitory effects on apoptosis than substitution of one cytoplasmic Ser residue did. There was no significant difference in the degree of inhibition between S5A/S27A, S2A/S27A, and S2A/S5A. In conclusion, Ser^2 and Ser^5 are critical in reverse signaling through mTNF by infliximab, whereas Ser^{27} works complementarily.

The finding that infliximab-induced apoptosis requires 12 hours from the beginning of stimulation prompted us to add CHX to examine whether newly synthesized protein(s) were involved in the induction of apoptosis. Twenty-four hours of stimulation with infliximab at 10 μg/mL resulted in 71.8% of Annexin V-positive cells in WT-mTNF transfected Jurkat cells without CHX, whereas CHX pretreatment dose dependently decreased the percentage of Annexin V-positive cells to 46.4% with CHX 0.5 μg/mL, 24.2% with 2.0 μg/mL, and 17.4% with 10 μg/mL (Figure 5*D*). These results indicate that the synthesis of novel protein is required for the induction of apoptosis.

We then analyzed the effect on apoptosis of cross-linking of cell surface etanercept (Figure 5*E*). Cross-linking of etanercept with anti-human IgG resulted in a slight increase in the percentage of Annexin V-positive/PI-negative early apoptotic cells from 4.32% to 12.91%, and this was less pronounced than that obtained with infliximab, as demonstrated in Figure 5*A*. There was no significant increase in the percentage of Annexin V/PI-positive cells, even after cross-linking. To rule out the possibility that death receptor was up-regulated by infliximab and then mediated apoptosis, we further investigated the cell-surface expression of TNF-RI, TNF-RII, and Fas on cells stimulated with infliximab, using flow cytometry. The expression of these molecules was unchanged after infliximab treatment (data not shown).

Infliximab Arrests G0/G1 Cell Cycle in WT mTNF-Transfected Jurkat Cells but Etanercept Does Not

We next investigated cell cycle perturbation as a possible mechanism of the suppression of cell proliferation induced by infliximab (Figure 6*A*). After 24 hours of treatment with a control Ab, rituximab, at 10 μg/mL, 54.0% of Jurkat cells were in G1 phase, and 38.1% of cells were in S phase. The distribution of the cell cycle was not altered after 48 hours in the presence of rituximab. On the other hand, treatment with infliximab 10 μg/mL for 24 hours resulted in an increase in proportion of G1 cells to 69.6% and a decrease in the proportion of S cells to 24.7%. After 48 hours of treatment with infliximab, the increase in the G1 population was more prominent, reaching 87.9%, and the S population had decreased to 6.2%, indicating that infliximab induced a G0/G1 arrest. In contrast, treatment with etanercept for 24 and 48 hours had no effect on the cell cycle. The proportion of sub-G1 cells, belonging to apoptotic cells, was 19.0% with infliximab, 4.0% with etanercept, and 4.0% with rituximab after 48 hours of treatment. We next investigated whether the 3 cytoplasmic Ser residues were essential for cell cycle G0/G1 arrest by infliximab in mTNF-transfected Jurkat cells, as in the case of apoptosis (Figure 6*B*). In Jurkat cells transfected with S2A/S5A/S27A, infliximab caused neither an increase in the G0/G1 population nor a decrease in the S population.

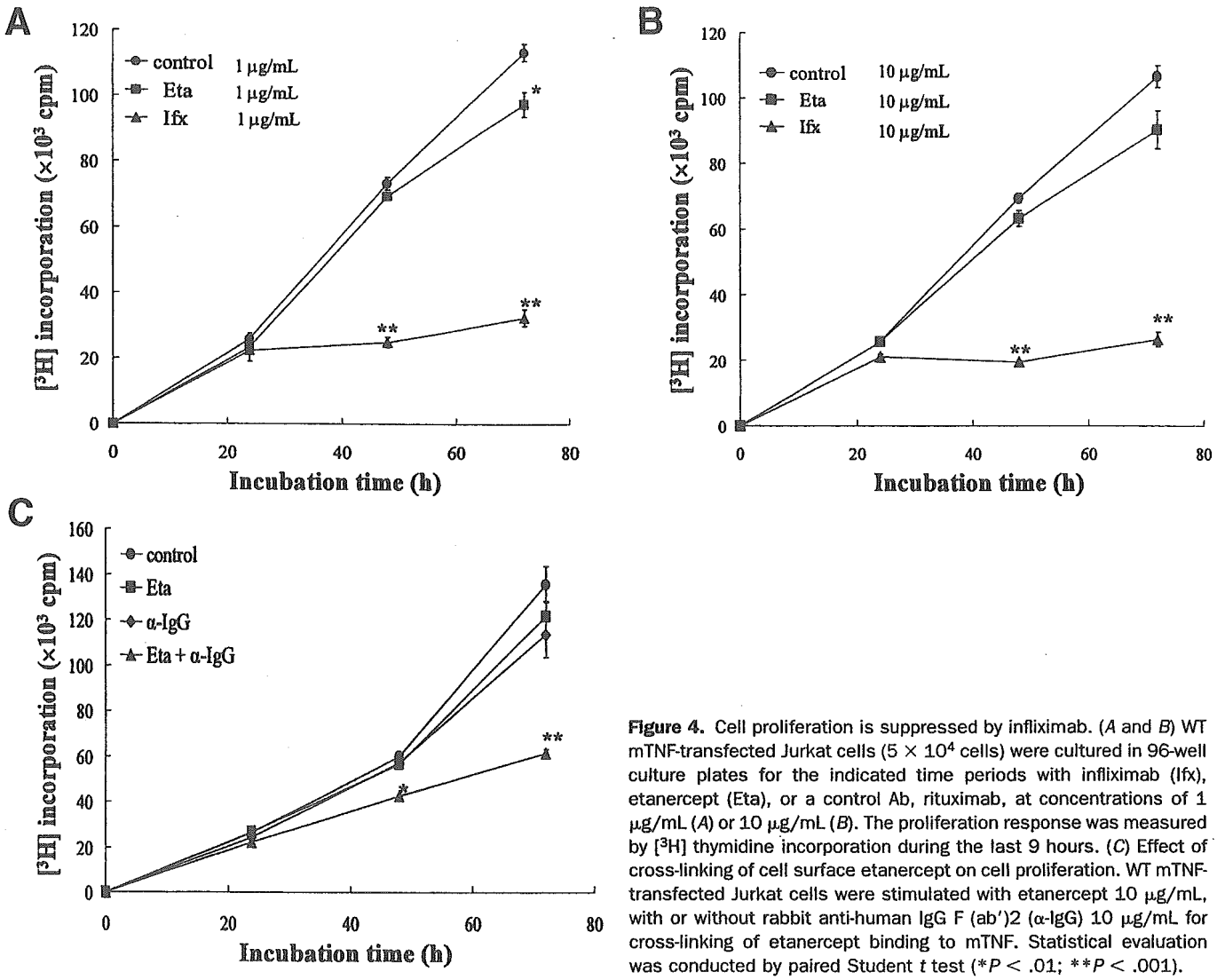


Figure 4. Cell proliferation is suppressed by infliximab. (*A* and *B*) WT mTNF-transfected Jurkat cells (5×10^4 cells) were cultured in 96-well culture plates for the indicated time periods with infliximab (Ifx), etanercept (Eta), or a control Ab, rituximab, at concentrations of 1 μg/mL (*A*) or 10 μg/mL (*B*). The proliferation response was measured by [^{3}H] thymidine incorporation during the last 9 hours. (*C*) Effect of cross-linking of cell surface etanercept on cell proliferation. WT mTNF-transfected Jurkat cells were stimulated with etanercept 10 μg/mL, with or without rabbit anti-human IgG F (ab')2 (α-IgG) 10 μg/mL for cross-linking of etanercept binding to mTNF. Statistical evaluation was conducted by paired Student *t* test (*$P < .01$; **$P < .001$).

Caspase Cascade Is Involved in Apoptosis Induced by Infliximab but not in Cell Cycle Arrest

To investigate further the pathway of apoptosis, we examined the effect of the broad caspase inhibitor zVAD-fmk on infliximab-induced apoptosis. Caspase-3 activation has been demonstrated by stimulation with infliximab of monocytes and activated lamina propria T lymphocytes.[31,32] The sub-G1 population of the cells stimulated by infliximab was reduced from 20.3% to 8.9% by pretreatment with z-VAD-fmk; this was comparable with the basal level of 8.0% apoptosis. This result suggests that infliximab-induced apoptosis required caspase activation (Figure 7*A*). On the other hand, infliximab-induced G0/G1 arrest was not inhibited by z-VAD-fmk. We next examined caspase activation by Western blot analysis. As shown in Figure 7*B*, activated fragments of caspase-3 were identified 24 hours after treatment with infliximab, whereas the control Ab, rituximab, had no effect on caspase-3 activation. Caspase-8 was not activated by treatment with infliximab or rituximab.

ROS Accumulation by Infliximab

We assessed the oxidization of DCFH-DA to determine whether infliximab induces ROS accumulation in mTNF-expressing cells. The fluorescent intensity of DCFH-DA increased after stimulation with infliximab 10 μg/mL. In contrast, no substantial increase was observed with etanercept (Figure 8*A*). In the kinetic assay, the peak of ROS accumulation by infliximab was 12 hours after stimulation (Figure 8*B*).

Bax, Bak, and p21$^{WAF1/CIP1}$ Are Up-regulated by Infliximab

Because p53 is a representative molecule that regulates both apoptosis and cell cycle arrest, we next examined

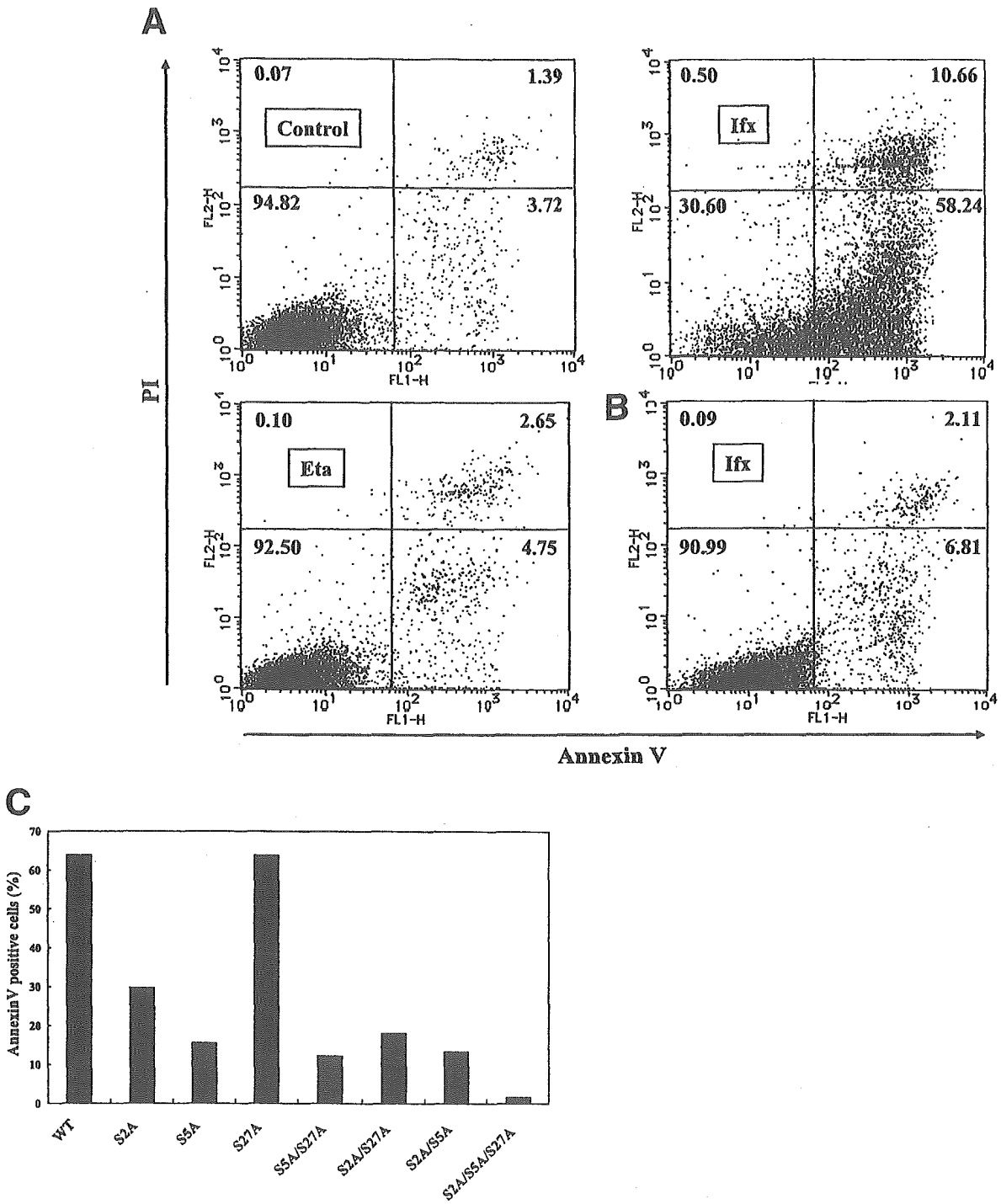
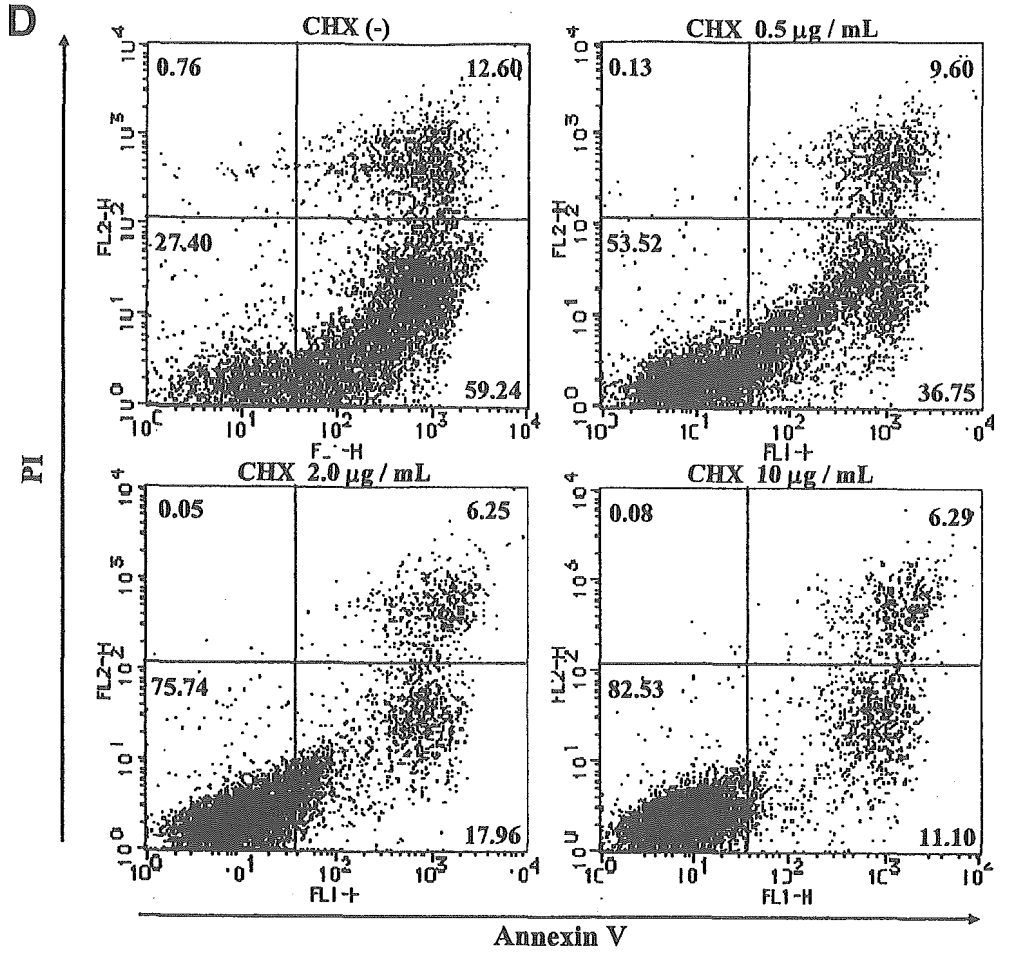


Figure 5. Ser residues in the cytoplasmic domain of mTNF are essential for infliximab-induced apoptosis in mTNF-transfected Jurkat cells. (*A*) WT mTNF-transfected Jurkat cells were treated with infliximab (Ifx), etanercept (Eta), or a control Ab, rituximab, at 10 μg/mL and cultured for 24 hours. The stimulated cells were stained with FITC-conjugated Annexin V and PI and were then analyzed by flow cytometry. Ten thousand cells were measured and plotted. The proportion of cells residing in each quadrant is expressed as a percentage. Results are representative of 3 independent experiments. (*B*) S2A/S5A/S27A mTNF transfectants were stimulated for 24 hours with infliximab 10 μg/mL and analyzed for apoptosis by the same method used for WT. Results are representative of 3 independent experiments. (*C*) WT and Ser-replaced mutant forms of mTNF transfectants were analyzed for apoptosis 24 hours after stimulation with infliximab 10 μg/mL. The proportions of Annexin V-positive cells in each transfectant after stimulation with infliximab are indicated as *solid columns*. (*D*) CHX effect on infliximab-induced apoptosis. WT mTNF transfectants were pretreated for 30 minutes with or without CHX at concentrations of 0.5, 2.0, or 10 μg/mL, followed by stimulation with infliximab 10 μg/mL. After 24 hours, cells were analyzed by flow cytometry for Annexin V-PI staining. (*E*) Effect of cross-linking of cell surface etanercept on apoptosis. WT mTNF transfectants were stimulated with etanercept 10 μg/mL, with or without rabbit anti-human IgG F(ab')2 (α-IgG) 10 μg/mL for cross-linking of etanercept binding to mTNF, followed by analysis by flow cytometry.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9.60
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CHX 2.0 μg / mL
0.05
6.25
75.74
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CHX 10 μg / mL
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82.53
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PI
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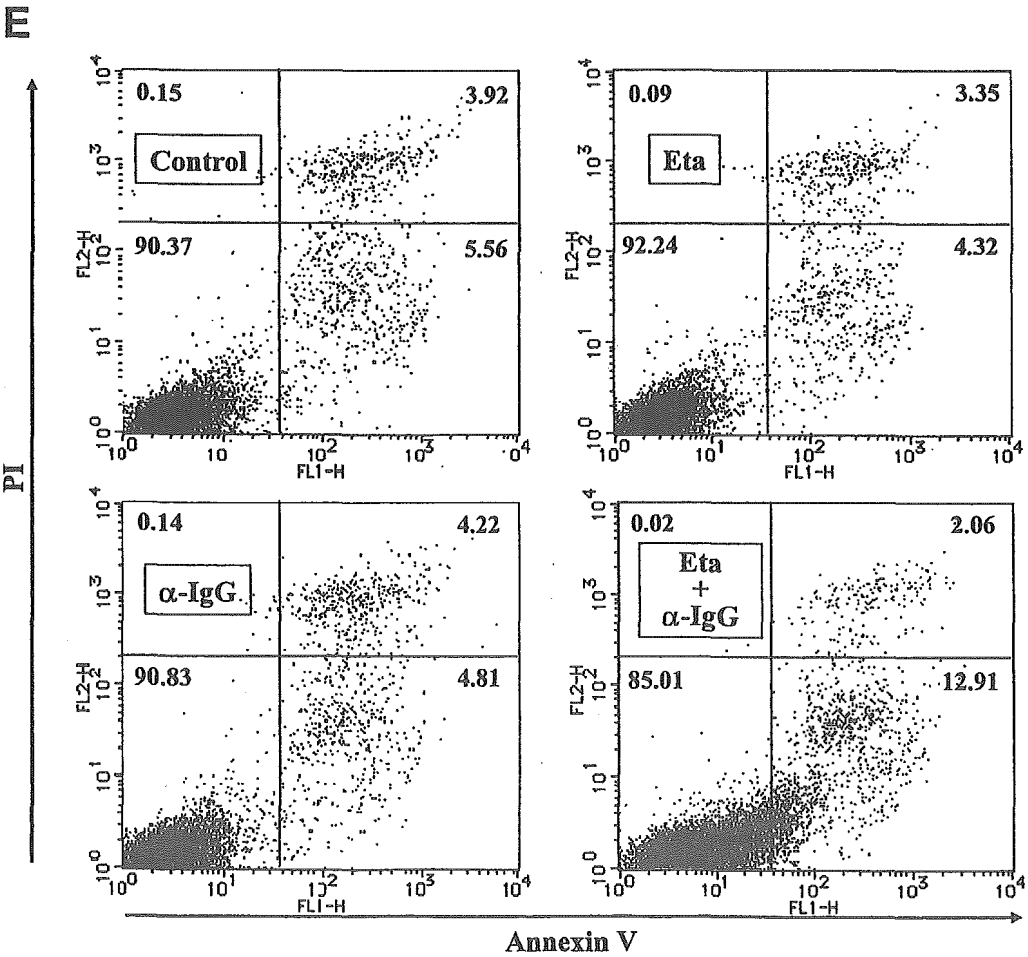
E
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3.92
Control
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5.56
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Eta
92.24
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90.83
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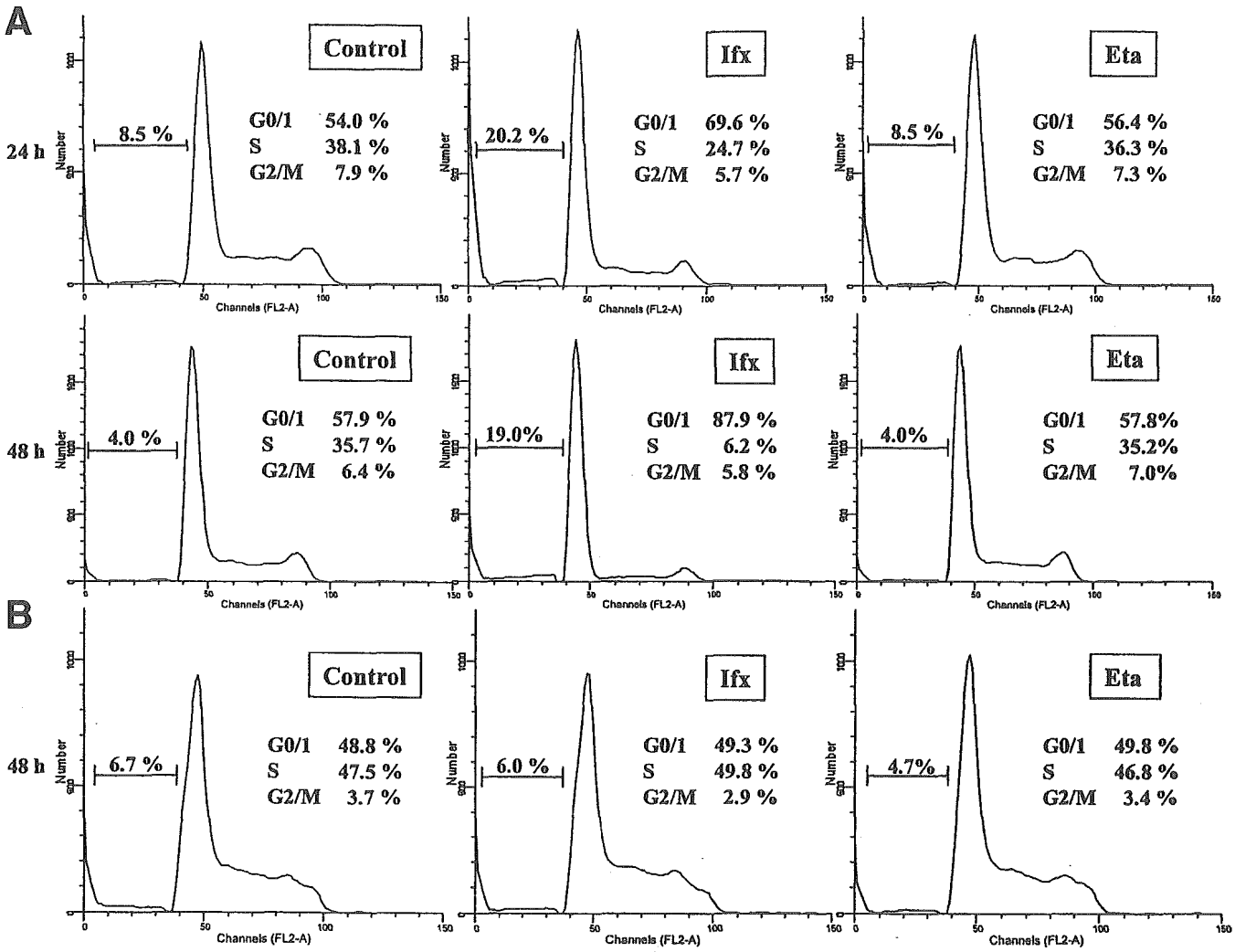

Figure 6. Ser residues in the cytoplasmic domain of mTNF are essential for infliximab-induced G0/G1 cell cycle arrest. WT (*A*) and S2A/S5A/S27A (*B*) mTNF-transfected Jurkat cells were incubated for 24 or 48 hours with infliximab (Ifx), etanercept (Eta),or a control Ab, rituximab, at 10 μg/mL. The cells were fixed in ice-cold 70% ethanol, stained with PI, and then analyzed for cell cycle distribution by flow cytometry (at least 20,000 cells). Data are plotted as DNA content (PI fluorescence) vs. relative cell numbers. The percentages of cells in the Sub-G1, G0/G1, S, and G2/M phases of the cell cycle are indicated. Results are representative of 3 independent experiments.

whether p53-inducible genes were involved in mTNF signaling. Bax and Bak have been reported to be transcriptionally regulated by p53 and to be essential for p53-mediated apoptosis.[33,34] As shown in Figure 8*C*, Bax and Bak proteins were up-regulated in WT mTNF-transfected Jurkat cells 6 hours after stimulation with infliximab 10 μg/mL. Stimulation with the same dose of etanercept did not have any effect on the expression of Bax and Bak until 24 hours (data not shown). We next examined $p21^{WAF1/CIP1}$, a cyclin-dependent kinase inhibitor at the G1/S cell cycle checkpoint that is also considered to be induced by p53.[35] Like Bax protein, $p21^{WAF1/CIP1}$ protein was up-regulated by stimulation with infliximab 10 μg/mL (Figure 8*C*) but not with etanercept (data not shown). Another cyclin-dependent kinase inhibitor, $p27^{KIP1}$ was not up-regulated by infliximab (data not shown).

JNK Pathway Is Essential for Infliximab-Induced Apoptosis and Cell Cycle Arrest but not for E-Selectin Induction

We next investigated whether the mitogen-activated protein kinase (MAPK) cascade was involved in infliximab-induced apoptosis and cell cycle arrest. JNK phosphorylation was observed as early as 30 minutes after the stimulation with infliximab, with a peak at 1.5 hours, which almost completely disappeared 6 hours after stimulation (Figure 9*A*). In addition, SP600125, an anthrapyrazolone inhibitor of JNK, inhibited infliximab-induced apoptosis, as assessed by the percentage of Annexin V-positive cells, in a dose-dependent manner (Figure 9*B*). At an SP600125 dose of 80 μmol/L, apoptosis was almost completely inhibited. Neither SB203580, an inhibitor of

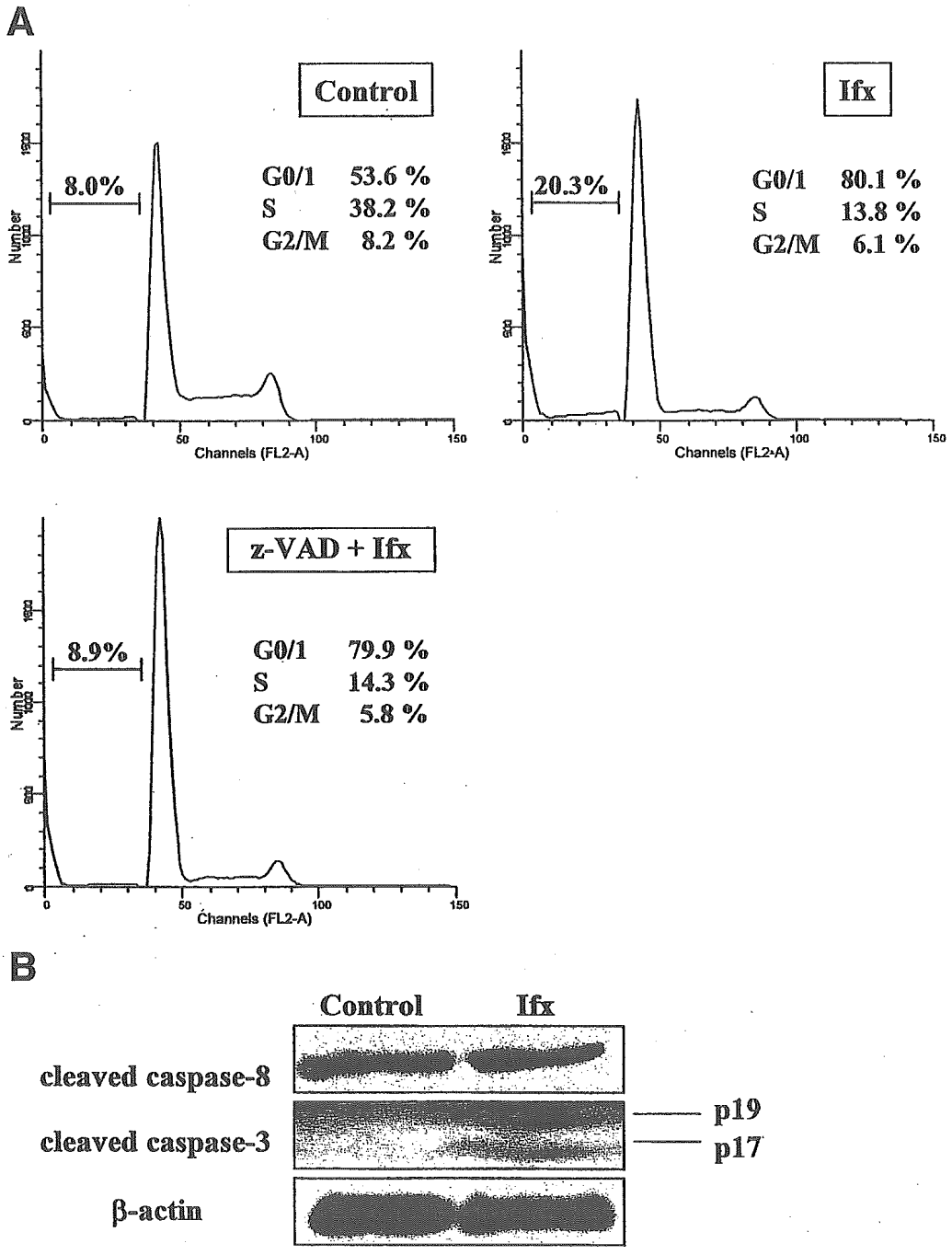

Figure 7. Caspase-3 is activated by stimulation with infliximab but is not involved in cell cycle arrest. (*A*) WT mTNF-transfected Jurkat cells were untreated or pretreated with 50 μmol/L of z-VAD-fmk for 30 minutes, followed by stimulation for 48 hours with infliximab (Ifx) or a control Ab, rituximab, at 10 μg/mL. Cells were stained with PI and the cell cycle distribution was analyzed using a flow cytometer. The percentages of cells in the Sub-G1, G0/G1, S, and G2/M phases of the cell cycle are indicated. Results are representative of 2 independent experiments. (*B*) WT mTNF-transfected Jurkat cells were stimulated for 24 hours with infliximab or a control Ab at 10 μg/mL. Stimulated cells were lysed in SDS sample buffer, and the expression levels of active forms of caspase-8 and caspase-3, as well as β-actin (control), were examined by Western blot analysis using rabbit anti-caspase-8 polyclonal Ab, rabbit anti-cleaved caspase-3 polyclonal Ab, and mouse anti-β-actin mAb.

p38, nor PD98059, an inhibitor of ERK, inhibited infliximab-induced apoptosis, even at concentrations of 100 μmol/L (data not shown). Infliximab-induced p21 protein up-regulation was also inhibited by SP600125 in a dose-dependent manner (Figure 9*C*). E-selectin induction by infliximab and etanercept was not inhibited by SP600125 at a concentration of 80 μmol/L (data not shown).

Discussion

In the present study, we have shown a number of novel findings with regard to the outside-to-inside (reverse) signal through mTNF by using mTNF-transfected Jurkat T cells: (1) mTNF induced apoptosis, cell cycle G0/G1 arrest, ROS, and IL-10; (2) cytoplasmic Ser residues of mTNF, S2, S5, and S27 are essential for these biologic effects; and (3) JNK activation followed by up-regulation of the molecules in p53 pathway is an important intracellular signaling event for apoptosis and cell cycle arrest. These effects were elicited by treatment of mTNF with infliximab but not with etanercept. On the other hand, E-selectin was similarly induced on the Jurkat T cells by infliximab and etanercept. This result

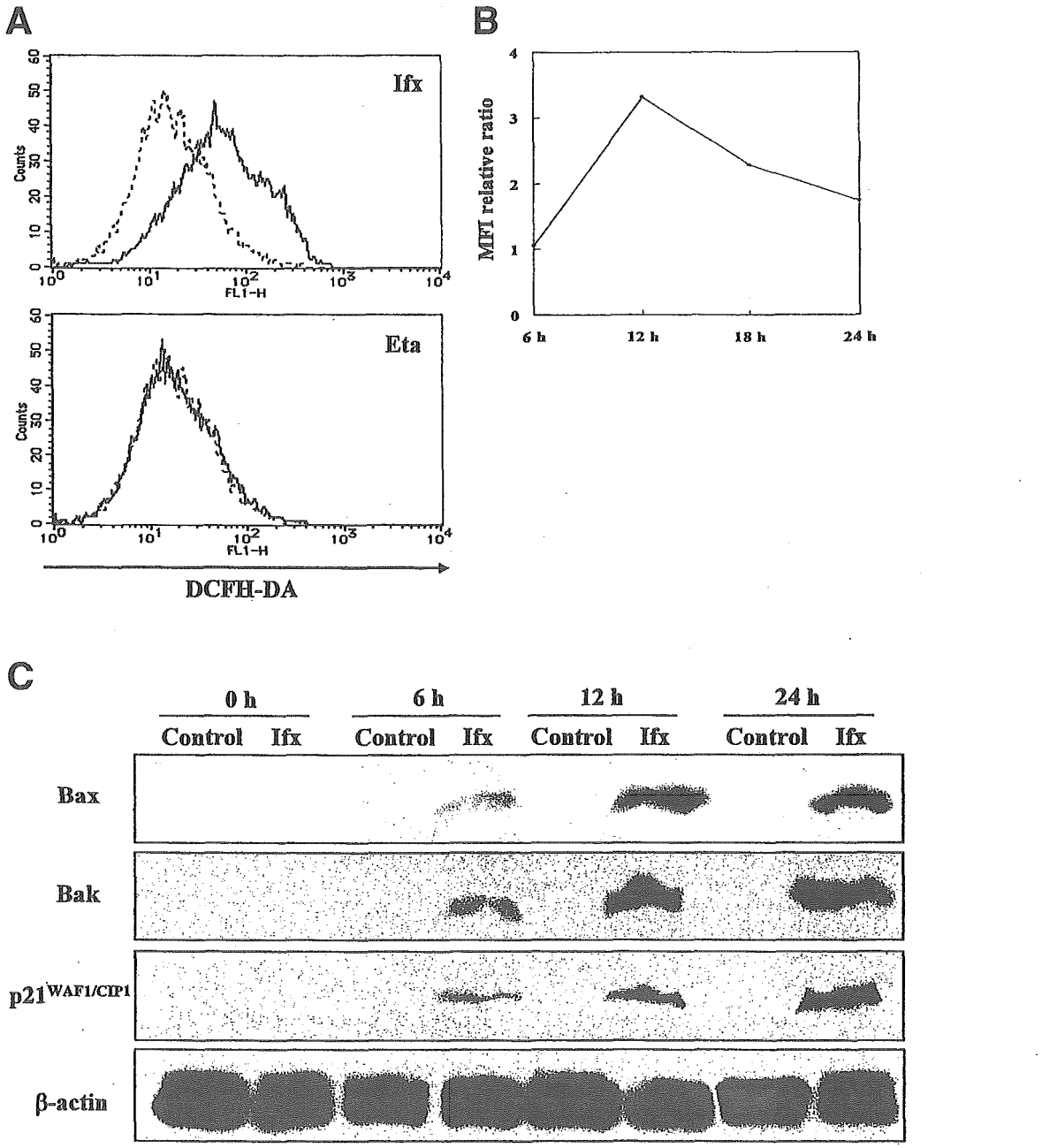

Figure 8. ROS accumulation and Bax, Bak, and $p21^{WAF1/CIP1}$ up-regulation are induced by infliximab but not by etanercept. (*A*) WT mTNF-transfected Jurkat cells were incubated with infliximab (Ifx), etanercept (Eta), or a control Ab, rituximab, at 10 μg/mL for the indicated time periods. The stimulated cells were incubated with DCFH-DA (5 μmol/L) for 30 minutes at 37°C. The fluorescence intensities of more than 10,000 cells were analyzed using a flow cytometer. The data on infliximab and etanercept are indicated as *solid lines*, and the data on the control Ab are indicated as *dotted lines*. (*B*) Kinetics of ROS production by stimulation with infliximab 10 μg/mL. Relative fluorescence intensities (infliximab vs. control Ab) are plotted. (*C*) WT mTNF-transfected Jurkat cells were stimulated with infliximab, etanercept, or a control Ab, rituximab, at 10 μg/mL, for the indicated time periods. The stimulated cells were lysed in SDS sample buffer, and protein-expression levels of Bax, Bak, $p21^{WAF1/CIP1}$, and β-actin (control) were examined by Western blot analysis using rabbit anti-Bax polyclonal Ab, rabbit anti-Bak polyclonal Ab, mouse anti-$p21^{WAF1/CIP1}$ mAb, and mouse anti-β-actin mAb.

confirms our previous observation that treatment of mTNF with rabbit anti-TNF-α polyclonal Ab resulted in E-selectin expression on Jurkat T cells and CD4+ human T cells.[16] Substitution of all the 3 cytoplasmic Ser residues by Ala residues, as well as inhibition of the JNK pathway, did not affect E-selectin expression by infliximab and etanercept. This indicates that the pathway for mTNF-mediated E-selectin expression is independent of the mTNF cytoplasmic Ser/JNK/p53-related pathway.

In contrast to the well-known biologic activities of mTNF as a ligand, reverse signaling through mTNF as a receptor is poorly understood in terms of both biologic

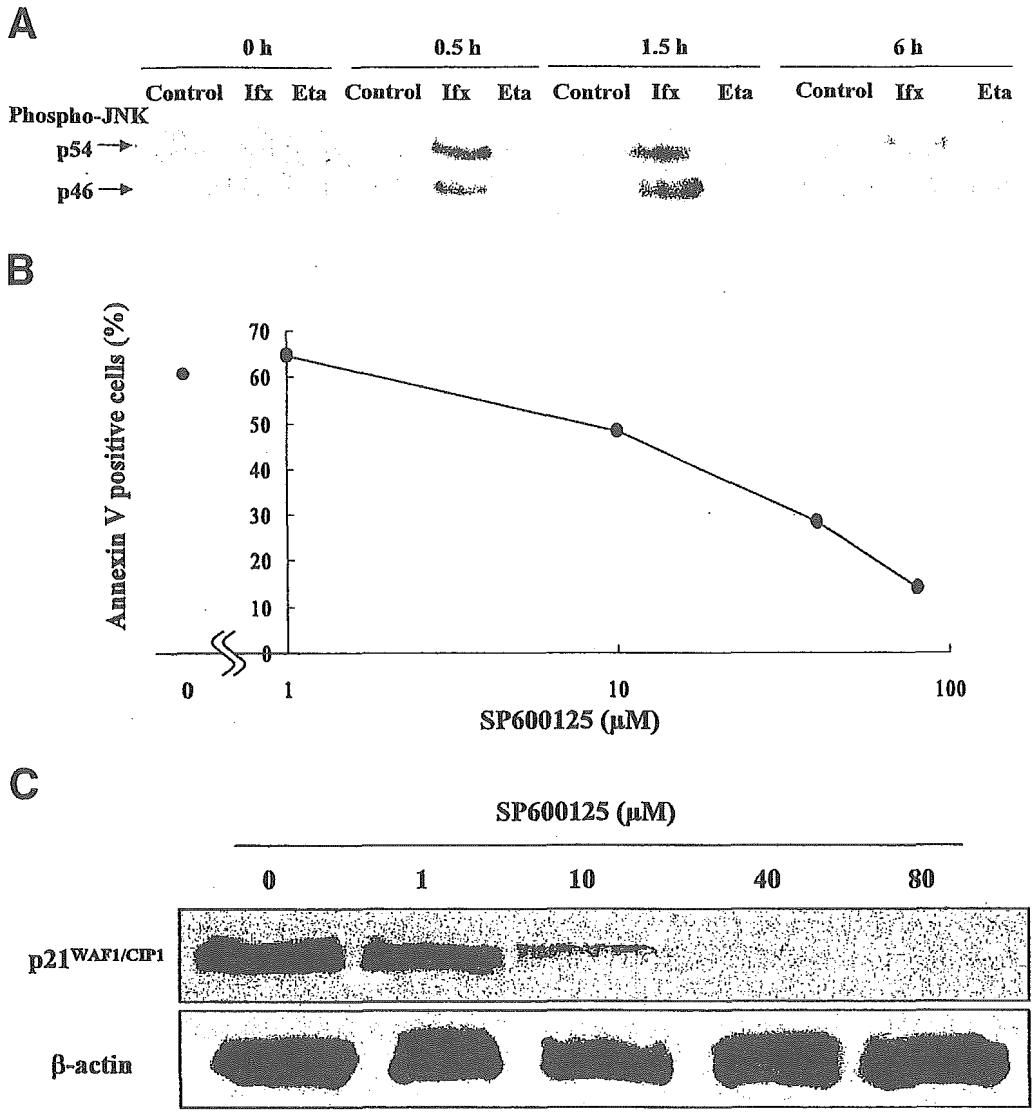

Figure 9. JNK activation is essential for infliximab-induced apoptosis and cell cycle arrest. (*A*) WT mTNF-transfected Jurkat cells were stimulated with infliximab (Ifx), etanercept (Eta), or a control Ab, rituximab, at 10 μg/mL for the indicated time periods. Stimulated cells were lysed in SDS sample buffer, and the phosphorylation of JNK was analyzed by Western blot using anti-phospho-SAPK/JNK (Thr183/Tyr185) polyclonal Ab. (*B* and *C*) WT mTNF-transfected Jurkat cells were pretreated for 30 minutes with 1, 10, 40, or 80 μmol/L of SP600125, a specific JNK inhibitor, followed by stimulation for 24 hours with infliximab 10 μg/mL. (*B*) Stimulated cells were stained with FITC-conjugated Annexin V and PI then analyzed by flow cytometry. The proportions of Annexin V-positive cells in each condition were plotted. (*C*) Stimulated cells were lysed in SDS sample buffer and protein-expression levels of $p21^{WAF1/CIP1}$ were examined by Western blot analysis using mouse anti-$p21^{WAF1/CIP1}$ mAb and mouse anti-β-actin mAb (control).

functions and essential intracellular signaling components. Recently, the reverse signaling mediated by mTNF has received increasing attention. The 2 kinds of TNF antagonists, infliximab and etanercept, equally neutralize the soluble form of TNF-α and are in fact equally effective against rheumatoid arthritis.[19–21,29] In contrast, only infliximab is effective against Crohn's disease but etanercept is not. This clinical effect might be caused by some additional effect of infliximab, such as reverse signaling, although in the study of Crohn's disease, the possibility that the dose of etanercept may not have been sufficient to see an effect is not completely ruled out. In addition, infliximab can induce long-term remission compared with its pharmacokinetics in rheumatoid arthritis and in Crohn's disease.[21,36] These findings indicate that TNF-α antagonists might have some cellular functions in addition to mere neutralization of soluble TNF-α. Infliximab was recently shown to induce apoptosis in PBMCs and activated T lymphocytes and in lamina propria T lymphocytes from active Crohn's disease in vivo and in vitro.[31,32] Because these experiments were performed on peripheral human T cells that express soluble and membrane forms of TNF-α, TNF-RI, and TNF-RII, it is difficult to conclude whether the mechanism of this apoptosis was related to reverse signaling in response to infliximab binding to mTNF or to neutralization of the effect of soluble and membrane forms of TNF on TNF-RI or TNF-RII. Our experimental condition is simple because only the uncleavable form of mTNF is expressed on human Jurkat T cells, which do not express TNF-RI and TNF-RII. More importantly, by introducing point mutations to the cytoplasmic Ser residues, we were able to identify specifically the mTNF-mediated outside-to-inside signal dependent only on the cytoplasmic domain of mTNF. To exclude the possibility that soluble TNF in the culture media might affect