Università degli Studi del Piemonte Orientale

"Amedeo Avogadro"

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(XX Ciclo)

IL RUOLO DI H4/ICOS NELLA RISPOSTA IMMUNITARIA

Responsabile scientifico:

Prof. Umberto Dianzani

Dottoranda:

Ilaria Seren Bernardone

I haven’t failed, I’ve found 10,000 ways taht don’t work

- *Thomas Edison*

Indice

Introduzione

Un'efficace risposta immunitaria adattativa coinvolge due principali gruppi di cellule: i linfociti e le cellule presentanti l'antigene (*antigen presenting cells,* APC).
I linfociti, come gli altri globuli bianchi, originano nel midollo osseo. Circolano nel sangue e nel sistema linfatico e risiedono in vari organi linfatici. Poiché producono ed esprimono recettori di superficie capaci di legare l'antigene, i linfociti sono i mediatori delle caratteristiche di specificità, diversità, memoria e discriminazione tra *self* e *not-self*. I linfociti si dividono in due principali popolazioni: linfociti T, responsabili della risposta cellulo-mediata, e linfociti B, responsabili della risposta umorale.
Le APC invece hanno il compito di garantire una fine regolazione dell'attivazione linfocitaria: infatti i linfociti sono in grado di riconoscere solo gli antigeni presentati dalle APC. Sono APC i macrofagi, le cellule dendritiche e i linfociti B. Esse esprimono in membrana molecole MHC di classe II e sono in grado di trasmettere un segnale costimolatorio per i linfociti T; le molecole MHC di classe II presentano ai linfociti T *helper* l'antigene (un peptide) dopo averlo captato per endocitosi dall'ambiente esterno e averlo processato attraverso il ciclo endocitico [Goldsby R.A. et al., 2000].

1. I linfociti T

I linfociti T nascono nel midollo osseo e maturano nel timo, dove esprimono i vari recettori di superficie che li caratterizzano.

1.1 Le molecole di superficie dei linfociti T

Il più importante complesso transmenmbrana sulla superficie dei linfociti T è costituito dal *T cell receptor* (TCR) associato a CD3: il TCR riconosce e lega l'antigene, mentre CD3 svolge la funzione di trasduzione del segnale. Il TCR è un eterodimero costituito dall'associazione di due catene proteiche α e β oppure γ e δ unite tra loro da ponti disolfuro. Le estremità amino-terminali delle due catene proteiche rappresentano la parte variabile della molecola, e si ripiegano a formare la regione del TCR destinata a legare l'antigene. A differenza delle immunoglobuline che riconoscono l'antigene come tale, il TCR riconosce l'antigene (in genere un peptide di otto-quindici amminoacidi) esclusivamente se associato con le molecole del complesso maggiore d'istocompatibilità (MHC). Una sequenza di residui amminoacidici idrofobici forma la porzione transmembrana. Una caratteristica singolare di tale regione è la presenza di residui carichi

positivamente che interagiscono con amminoacidi carichi negativamente presenti nella porzione transmembrana dei polipeptidi del complesso CD3. Le catene α e β possiedono code C-terminali intracellulari di lunghezza pari a 5-12 aminoacidi, troppo brevi per possedere un'attività enzimatica intrinseca: per questo sono necessarie altre molecole associate al TCR per permettere la trasduzione del segnale. Il complesso CD3 è costuito dalle catene γ, δ, ε, ζ e η: le prime tre hanno un dominio extracellulare immunoglobulinico e contengono un singolo motivo ITAM (*immunoreceptor tyrosine-based activation motif*) nella coda citoplasmatica. Il motivo ITAM è formato da circa 26 residui amminoacidici per lo più non conservati, in cui due sequenze Tyr-X-X-Leu sono separate da 6-8 residui. I residui tirosinici delle ITAM sono fosforilati, in risposta al riconoscimento dell'antigene da parte del TCR. Questo consente alle ITAM di legare proteine contenenti un dominio SH2, dando avvio ad una cascata d'eventi intracellulari. La catena ζ, presente come omodimero o come eterodimero se associato alla sua variante di *splicing* η, ha una ridotta regione extracellulare e contiene tre motivi ITAMs nella coda citoplasmatica.

Esistono poi una serie di molecole accessorie che hanno il compito o di rafforzare l'adesione dei linfociti T ad altri tipi cellulari, o di trasdurre segnali che potenziano la risposta dei linfociti T, o di regolare la migrazione dei linfociti T verso i vari distretti anatomici. I corecettori CD4 e CD8 si legano a regioni non polimorfe delle molecole MHC e in questo modo favoriscono l'adesione dei linfociti T alle APC o alle cellule bersaglio e partecipano agli eventi precoci di trasduzione del segnale che si verificano a seguito del riconoscimento da parte dei linfociti T dei complessi MHC-peptide espressi sulle APC. La trasduzione del segnale è in parte da attribuirsi all'associazione, a livello della coda citoplasmatica, con la tirosina-chinasi Lck. In base all'espressione di questi corecettori è possible distinguere due sottopopolazioni di linfociti T: T helper, che esprimono CD4, e T citotossici, che esprimono CD8.

Nell'uomo esistono due categorie di molecole MHC: quelle di classe I comprendono HLA-A, HLA-B e HLA-C (riconosciute da CD8), mentre quelle di classe II HLA-DR, HLA-DP e HLA-DQ (riconosciute da CD4). Queste molecole sono caratterizzate da un elevato grado di polimorfismo nella popolazione, giustificato dal fatto che ciascuna molecola MHC ha una relativa selettività di legame con i peptici antigenici.

1.2 Le molecole costimolatorie

L'attivazione dei linfociti T rappresenta un evento centrale nella generazione della risposta immunitaria e ad essa consegue un'espansione clonale delle cellule immunocompetenti con l'acquisizione di funzioni effettrici [Janeway et al., 1993; Janeway et al., 1994; Dianzani et al., 1995]. Secondo il modello d'attivazione classico (ipotesi del doppio segnale [Bretscher P.A. et al., 1999]), i linfociti T necessitano, affinché siano indotte proliferazione e differenziamento in cellule effettrici, di due distinti segnali extracellulari: il primo e' costituito dal legame del TCR e dei corecettori CD4 e CD8 al complesso MHC-peptide (presente sulla superficie delle APC); il secondo è fornito da molecole costimolatorie, i cui ligandi si trovano anch'essi sulle APC [Harding F.A. et al., 1992].
L'esposizione dei linfociti ai complessi MHC-antigene in assenza del secondo segnale accessorio induce uno stato di anergia clonale o morte cellulare programmata.
Le molecole costimolatorie modulano, potenziando o frenando, la risposta del TCR all'antigene: in particolare i costimolatori positivi abbassano il livello di soglia di attivazione linfocitaria, definito dal numero di TCR che devono essere stimolati per avere una risposta biologica. In condizioni di basse concentrazioni di antigene o di antigeni con scarsa capacità immunogena, la costimolazione è essenziale nell'innescare l'attivazione linfocitaria [Viola et al.,1996].
Molte molecole costimolatorie appartengono a due superfamiglie: quella delle immunoglobuline (comprende CD28, CTLA4, ICOS, PD-1 e BTLA) e quella del TNFR/TNF (comprende, tra le altre, CD40L, OX-40, CD27) [Chambers et al., 1999].
Il mio progetto di dottorato è stato incentrato sullo studio di ICOS e del suo ligando B7h.

1.2.1 La famiglia CD28

Le molecole della famiglia del CD28 sono caratterizzate da un dominio IgV-like extracellulare, da una porzione transmembrana e da una coda citoplasmatica e comprendono CD28, CTLA-4 (CD152) e ICOS che mappano, nell'uomo, nella regione cromosomica 2q33; questa colocalizzazione suggerisce che i tre geni siano originati da duplicazione genica [Sharpe et al., 2002].
Queste tre molecole sono espresse in membrana come omodimeri, grazie a legami disolfuro tra residui di Cisteina nella regione extracellulare in posizione membrano-prossimale. Nella porzione intracitoplasmatica presentano un motivo comune: Tyr-Xaa-Xaa-Met (Tyr-Met-Asn-Met per CD28, Tyr-Met-Phe-Met per ICOS, e Tyr-Val-Lys-Met per CTLA-4). La fosforilazione della Tirosina in questi motivi permette l'associazione con la subunità adattatrice p85 di PI3K; CD28 può inoltre legare Grb-2 tramite il residuo Asn,

responsabile dell'esclusiva capacità di indurre la produzione di IL-2, e tramite domini ricchi in Prolina le chinasi Itk (IL-2 inducible T-cell kinase) ed Lck. Anche CTLA-4, a differenza di ICOS, presenta domini ricchi in Prolina ma un ligando per questi non è stato ancora individuato [Sharpe et al., 2002; Prasad et al., 1994; Schneider].

CD28 e CTLA-4 presentano lo stesso motivo MYPPPY nella porzione extracellulare, che è responsabile dell'interazione con i ligandi, B7.1 (CD80) e B7.2 (CD86); ICOS, presenta invece il motivo FDPPPF, responsabile del legame con il suo unico recettore, B7h (B7rp1, GL50, B7H2, LICOS) [Schwartz et al., 2001; Wang et al., 2002].

Nonostante l'analogia a livello aminoacidico, le tre molecole differiscono da un punto di vista funzionale. CD28 ed ICOS mediano segnali stimolatori per il linfocita, in sinergia o in momenti e microambienti diversi della risposta immunitaria; CTLA-4, invece, invia segnali inibitori che tendono ad attenuare la risposta linfocitaria.

CD28 è espresso costitutivamente sulla superficie del 90% dei linfociti T_H $CD4^+$ e del 50% dei $CD8^+$ e agisce in sinergia con i segnali mediati dal TCR, modulando la regolazione genica indotta dal solo TCR, e potenziando le risposte T all'antigene [Riley et al., 2002;]. L'effetto di tale stimolo porta a proliferazione cellulare, grazie alla forte induzione di IL-2, ed ad acquisizione di funzioni effettrici, ma anche alla prevenzione dell'apoptosi o dell'induzione di anergia che si possono verificare in risposta al solo stimolo antigenico. Non è chiaro se CD28 sia in grado di indurre attivazione di geni diversi da quelli regolati dal TCR, tramite una via del segnale indipendente, o se il suo ruolo sia dedicato esclusivamente all'amplificazione/modulazione del primo segnale.

CTLA-4, a differenza di CD28, è espresso in seguito ad attivazione del linfocita, e per la sua azione inibitoria sono stati proposti diversi meccanismi, tra questi: competizione per i ligandi con CD28 (per i quali ha maggiore affinità), induzione di un *signaling* negativo (grazie al legame della fosfatasi SHP2), e induzione della disgregazione dei *raft* lipidici assemblati nella sinapsi immunologica.

Infine, sono stati recentemente proposti un meccanismo d'inibizione indipendente dall'interazione col ligando, e l'induzione di segnali negativi nelle APC tramite i recettori B7 (*reverse signaling*) [Grohmann et al., 2002].

H4/ICOS rappresenta il terzo membro della famiglia del CD28. È espresso in seguito all'attivazione linfocitaria da linfociti $CD8^+$ e $CD4^+$, come CTLA-4, ed intensifica tutte le risposte basali dei linfociti T all'antigene: proliferazione, secrezione di citochine, espressione di molecole che mediano l'interazione cellula-cellula. L'interazione di ICOS

con B7h gioca inoltre un ruolo importante nella cooperazione linfociti B/linfociti T [Rudd et al., 2003].

PD-1 e BTLA sono gli ultimi membri della famiglia del CD28 ad essere stati caratterizzati; questi mostrano una forte omologia di sequenza tra loro, e sono localizzati nei cromosomi 2q37 e 3q13 rispettivamente.

PD-1 (*Programmed Death Gene-1*) è un recettore monomerico, e come CTLA-4, inibisce la proliferazione mediata dall'attivazione del TCR nei linfociti T e la produzione di citochine; è espresso da linfociti T helper e citotossici, da linfociti B, da monociti attivati, ed a bassi livelli da NKT. Lega due recettori appartenenti alla famiglia di B7, PD-L1 (B7-H1), espresso da cellule della linea ematopoietica e non, e PD-L2 (B7-DC), espresso soltanto da macrofagi e DCs. La funzione inibitoria nei linfociti T avviene in seguito al reclutamento delle fosfatasi SHP1 e SHP2 tramite il motivo ITSM (*immunoreceptor tyrosinebased switch motif*) [Tseng et al., 2001; Zhang et al., 2004]

BTLA (*B and T lymphocyte attenuator*), anch'esso monomerico, è espresso in seguito ad attivazione dai linfociti T, e da linfociti B quiescenti o attivati. Il meccanismo dell'inibizione mediata da BTLA è probabilmente da attribuirsi, come per PD-1, al reclutamento delle fosfatasi SHP1 e SHP2. Il ligando per questo recettore non è ancora noto [Watanabe et al., 2003] (Fig.1).

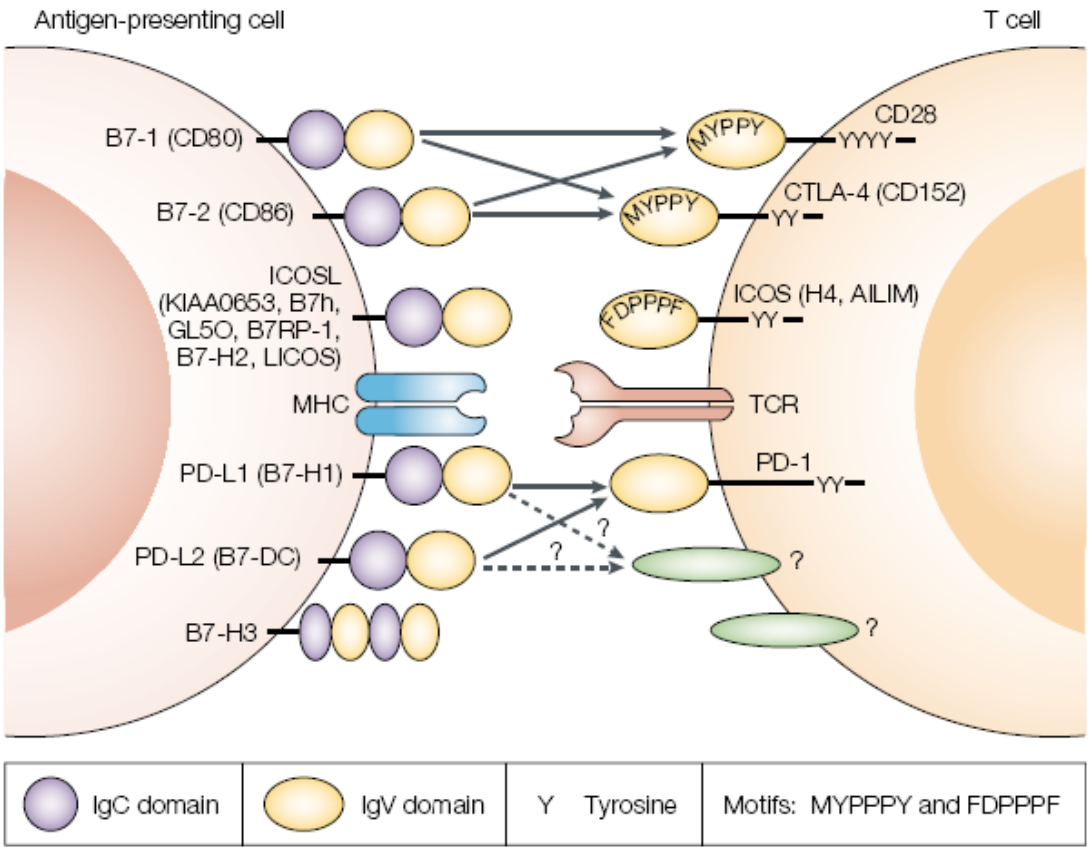

Figura 1 - Rappresentazione schematica delle famiglie B7 e CD28 [da Sharpe AH, Freeman GJ. The B7-CD28 superfamily. 2002. *Nat. Rev. Immunol.* 2:116-26.].

1.2.2 La molecola costimolatoria H4/ICOS

H4 è stata inizialmente identificata nel nostro laboratorio sia nel topo sia nell'uomo [Redoglia V. et al., 1996; Buonfiglio D. et al., 1999]. Kroczek e coll. hanno successivamente descritto il clonaggio nell'uomo di una nuova molecola costimolatoria, denominata ICOS (*i*nducible T cell *cos*timulator), espressa selettivamente da linfociti T attivati [Hutloff A. et al., 1999]. Nel nostro laboratorio è stato successivamente dimostrato che H4 ed ICOS sono la stessa molecola [Buonfiglio D. et al., 2000].

Cellule ICOS positive sono presenti negli organi linfoidi secondari, dove interagiscono con le cellule dendritiche e i linfociti B, nonché nel sangue periferico in seguito all'attivazione.

ICOS mappa nella regione cromosomica 2q33, come CD28 e CTLA-4 (Fig. 2).

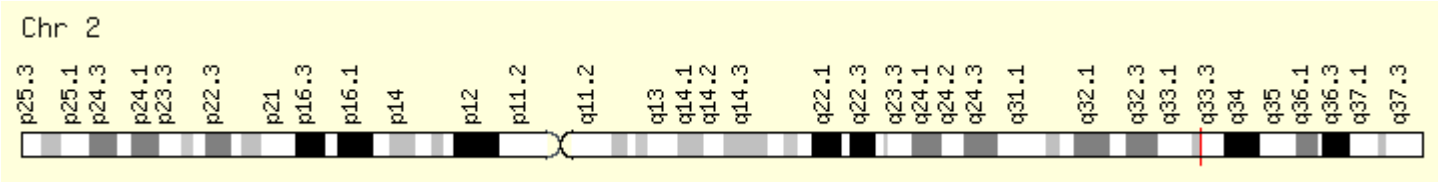

Figura 2 - Localizzazione cromosomica di H4/ICOS

La sequenza aminoacidica di ICOS mostra il 24% di somiglianza e il 39% di identità con CD28; sulla base dell'omologia di sequenza è stato proposto che ICOS rappresenti un terzo membro della famiglia molecolare di CD28, insieme a CD28 stesso ed a CTLA-4. A differenza di CD28, che è espresso costitutivamente sulla superficie dei linfociti T, ICOS viene espresso solo in seguito all'attivazione linfocitaria. La molecola è già espressa 24 ore dopo l'attivazione, tuttavia il picco di espressione viene raggiunto dopo 6 giorni; successivamente l'espressione della molecola si riduce gradualmente.

Pur appartenendo alla stessa famiglia, CD28 e ICOS si differenziano in alcuni importanti aspetti. Entrambe inducono la sintesi di citochine (IL-4, IL-5, IL-10, IFN-γ, TNF-α), ma soltanto CD28 induce forte secrezione di IL-2 [Hutloff A. et al., 1999; Yoshinaga S.K. et al., 1999]. Inoltre ICOS non lega i ligandi di CD28 e CTLA-4, B7-1 (CD80), e B7-2 (CD86), ma lega una nuova molecola chiamata B7-h (detta anche B7R-P1 o GL50), espressa costitutivamente da macrofagi, linfociti B e cellule dendritiche [Ling V. et al., 2000].

ICOS è una molecola transmembrana di tipo I, ed è espressa in forma di monomero o di omodimero; è costituita da 199 amminoacidi e la forma dimerica è probabilmente data da legami disolfuro tra residui di cisteina nella regione extracellulare in posizione membrano-prossimale; la forma dimerica ICOS ha un peso molecolare apparente di 55-60 kDa, mentre le singole catene di 27-29 kDa.. Ogni catena ha una coda citoplasmatica di 35 amminoacidi, una regione transmembrana di 23 amminoacidi, ed una regione extracellulare di 141 amminoacidi con un singolo dominio immunoglobulinico stabilizzato da due cisteine conservate in posizione 42 e 109 [Coyle J. et al., 2000].

H4/ICOS intensifica tutte le risposte basali dei linfociti T all'antigene: proliferazione, secrezione di citochine, espressione di molecole che mediano l'interazione cellula-cellula.

1.3 La sinapsi immunologica

E' stato proposto un modello di costimolazione, che prevede la formazione di una "sinapsi immunologica", o "SMAC" (*supramolecular activation cluster*), a livello del punto di contatto tra APC e cellula T. Secondo questa teoria l'attivazione del linfocita comporta l'innesco di

un processo dinamico di reclutamento di complessi recettoriali verso lo SMAC [Dustin M.L. et al., 1999]. Questo modello pone l'accento sull'esistenza di aree differenziate della membrana plasmatica, dette *raft* lipidici o GEM (*glycosphingolipid-enriched microdomains*), che sono fortemente rappresentate nello SMAC.

I *raft* lipidici sono microdomini dispersi nel mosaico fluido dei glicerofosfolipidi di membrana, e sono arricchiti in colesterolo, glicosfingolipidi e glicoproteine leganti GPI.

Le caratteristiche stesse dei raft lipidici, cioè la componente glicolipidica e la presenza di colesterolo, portano alla formazione di regioni di aumentata rigidità di membrana che, durante l'attivazione linfocitaria contribuiscono a mantenere il TCR impegnato nel legame con le molecole MHC. Dati sperimentali hanno dimostrato che i *raft* lipidici sono ricchi di molecole coinvolte nella trasduzione del segnale (LAT, PAG e PTK della famiglia Src), e che il TCR ne entra a far parte durante l'attivazione.

Secondo questo modello la struttura simil-sinaptica formatasi creerebbe una sorta di filtro molecolare capace di selezionare, in base alle dimensioni, le molecole coinvolte nel contatto cellula T/APC. Questo tipo di selezione fisica permetterebbe la regolazione dell'attivazione linfocitaria attraverso la modulazione delle componenti della zolla recettoriale.

Nel centro della sinapsi si accumulano il TCR, Lck, Fyn e PKCθ, mentre nella zona periferica della sinapsi si accumulano LFA-1, CD4, CD28, CD45.

La costimolazione ha dunque luogo in un'area definita della membrana, in cui si ha un aumento della concentrazione locale di molecole coinvolte nelle interazioni cellula-cellula e di trasduttori di segnali citoplasmatici [Xavier R. et al., 1999].

Recentemente è stato dimostrato che la costimolazione mediata da CD28 può innescare la riorganizzazione dei *raft* in corrispondenza del punto di contatto tra APC e cellula T. E' stato inoltre proposto un meccanismo per spiegare il ruolo di CD28 nel rinforzare lo stimolo dato dal complesso peptide-MHC al linfocita.

Secondo questo modello, l'ingaggio di CD28, consente l'iniziale adesione tra APC e linfocita e, contemporaneamente, attiva una via di trasduzione in grado di indurre la riorganizzazione dell'actina citoscheletrica. La connessione dei *raft* lipidici al citoscheletro potrebbe spiegare la riorganizzazione dei microdomini di membrana mediata da CD28. La riorganizzazione dei *raft*, e delle molecole associate, indurrebbe un'interazione più stabile tra TCR e complesso peptide-MHC all'interno della sinapsi immunologica [Viola A. et al, 1999]. Mentre la visione convenzionale prevede che ogni tipo di recettore generi un suo distinto segnale e che i singoli segnali siano poi armonizzati nel nucleo per regolare la

trascrizione, il nuovo modello ipotizza che la sinapsi immunitaria moduli ed amplifichi il segnale trasdotto tramite il TCR agendo a monte rispetto al nucleo.

1.4 Il signaling di H4/ICOS

La frazione citoplasmatica di H4/ICOS contiene un motivo Tyr-X-X-Met, comune a CD28 e CTLA4. In particolare H4/ICOS presenta la sequenza Tyr-Met-Phe-Met. Questo motivo, in seguito alla fosforilazione del residuo tirosinico, costituisce un sito di legame per proteine con dominio SH2. La specificità del legame è invece determinata dai residui adiacenti. Proprio in questo modo H4/ICOS e CD28 interagiscono con la subunità p85 della fosfatidilinositolo3 chinasi (PI3K) [Rudd C.E. et al., 2003; Prasad K.V.S. et al., 1994]. PI3K è particolarmente nota per il suo reclutamento da parte dei recettori per fattori di crescita. A differenza di CD28 però H4/ICOS non è in grado di legare Grb2 (*growth factor receptor bound protein 2*) perchè priva del residuo di Asparagina (Asn) nello stesso motivo. Un'altra differenza di H4/ICOS rispetto a CD28 e CTLA4 è l'assenza di specifici residui di Prolina (Pro) (motivo P-X-X-P) leganti i domini SH3 di Grb2 e LCK: proprio questa differenza sembrerebbe spiegare il mancato effetto di H4/ICOS sulla produzione di IL2, tramite la via di JNK.

L'unico interattore finora noto di H4/ICOS è quindi PI3K attraverso la quale attiva le vie di trasduzione di PDK, PKB e GSK3, legati a traduzione proteica, metabolismo cellulare e apoptosi. Inoltre PI3K sembrava svolgere una funzione chiave nella motilità delle cellule del sistema immunitario, e non, attraverso la sua azione sul riarrangiamento del citoscheletro. L'attivazione di PI3K induce la produzione di PIP3 che lega i domini PH (Plekstrin Homology), presenti per esempio nella proteina PDK-1 kinase, che attiva Akt1. La via di trasduzione PI3K/Akt è noto essere coinvolta nella chemiotassi dei neutrofili [Heit B.et al., 2002].

ICOS sembra essere responsabile del riarrangiamento citoscheletrico in linfociti T attivati non solo attraverso la via di PI3K/Akt, ma anche attraverso quella di Rho/ROCK [Nukada Y. et al, 2006].

1.5 I linfociti Th17

La risposta immunitaria adattativa mediata dai linfociti Th CD4+ può dimostrarsi molto eterogenea, a causa dell'esistenza di diverse sottoclassi caratterizzate dalla produzione di differenti *pattern* citochinici. [Annunziato F. et al., 2008]

I Th1 producono IFN-γ e svolgono un ruolo fondamentale nella difesa dai microorganismi intracellulari. Originano in risposta a IL-12 e IFNs prodotti da DC (*Dendritic Cells*) ed NK.

I Th2, che producono IL-4, IL-5, IL-9 e IL-13 ma non IFN-γ, intervengono nella risposta a parassiti gastrointestinali e nelle allergie. Il loro differenziamento è polarizzato da IL-4 (in assenza di IL-12).

I linfociti Th17 rappresentano una nuova sottoclasse di linfociti T CD4+ effettori, l'unica in grado di produrre IL-17A ed IL-17F in associazione ad altre citochine. Sono stati descritti sia nell'uomo che nel topo, anche se nelle due specie sembrano esistere alcune differenze.

I Th17 umani esprimono abbondantemente CCR6 (CC *chemokine receptor* 6) e CD161 (l'equivalente del NK1.1 murino) e sembrano originare in risposta alle citochine IL-1β e IL-23 da una sottoclasse di precursori delle cellule T CD161+CD4+ che si ritrova nel timo e nel sangue del cordone ombelicale. Tali cellule esprimono costitutivamente il recettore RORγt (Th17-*driving transcription factor retinoic acid-related orphan receptor*) e IL-23R. Topi KO per il gene *Rorc*, che codifica per RORγt, non rispondono alla stimolazione di IL-23, hanno un numero ridotto di Th17 e sono resistenti alle malattie autoimmuni. Possono anche differenziarsi in Th1 e Th2 in condizioni polarizzanti appropriate.

TGFβ non agisce direttamente sul differenziamento in Th17, ma indirettamente lo favorisce inibendo il differenziamento in Th1. I Th17 umani hanno una scarsa capacità proliferativa e una scarsa azione citotossica. Sono stati evidenziati cloni doppi positivi IFN-γ+IL-17+, ma nessuno doppio positivo per IL-4 e IL-17.

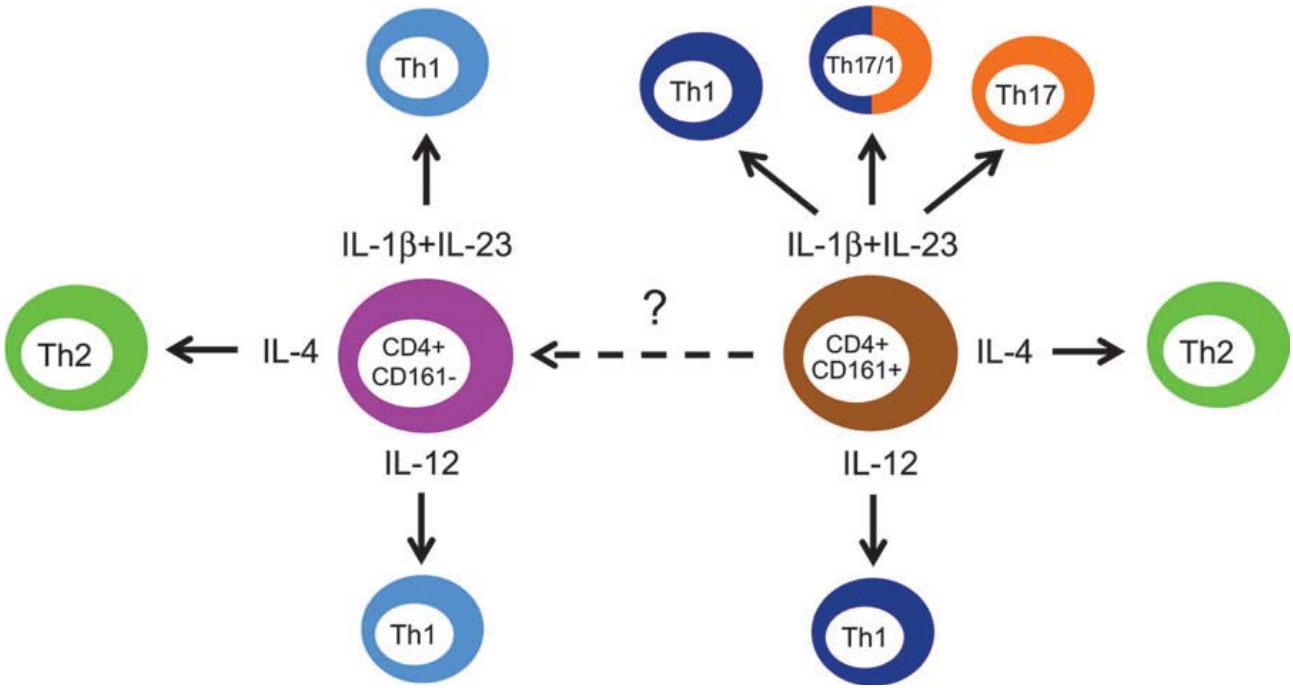

Fig.3 - Differenziamento/polarizzazione di Th17

[Annunziato F., 2008. *International Immunology*, Vol. 20, No. 11, pp. 1361–1368]

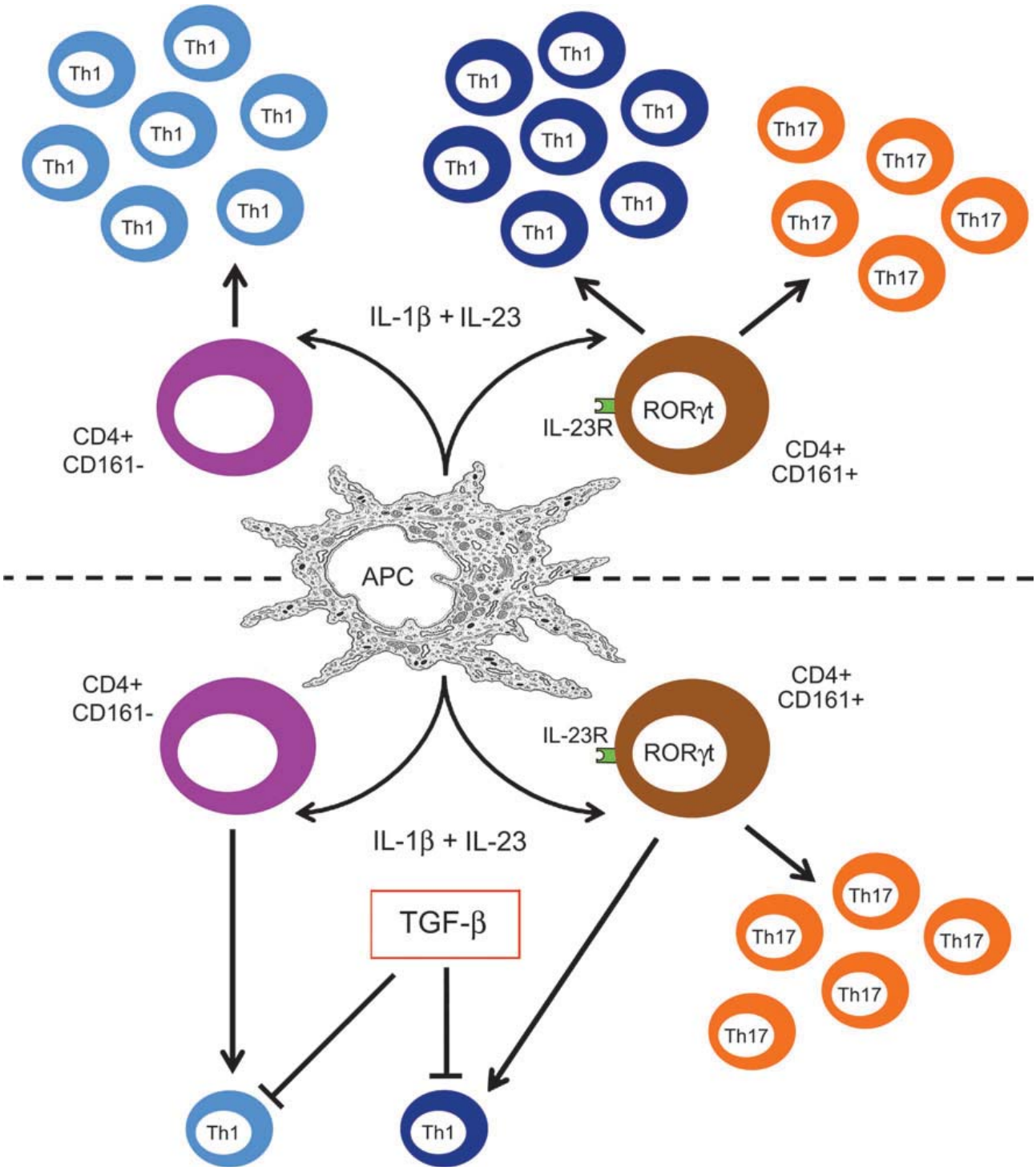

Fig.4 - Differenziamento/polarizzazione di Th17

[Annunziato F., 2008. *International Immunology*, Vol. 20, No. 11, pp. 1361–1368]

Nell'uomo Th17 e Th1 contribuiscono alla patogenesi di malattie autoimmunitarie e infiammatorie croniche (mentre nel topo in alcuni casi i Th1 possono svolgere un'azione protettiva). Fino ad ora la correlazione tra Th17 e malattie è stata riconosciuta con sicurezza solo nel caso della HIES (sindrome di Job o da iper-IgE). Mutazioni dominanti

negative al dominio legante il DNA di STAT3 sono state correlate a tale sindrome: tali mutazioni causano inefficiente differenziamento e quindi ridotti livelli di Th17. Poiché i Th17 sono ritenuti importanti nella risposta a funghi e batteri extracellulari, la loro assenza correla con la aumentata suscettibilità a infezioni che caratterizza la HIES.

Al contrario, il ruolo patogenico dei Th17 in malattie autoimmuni e infiammatorie croniche necessita ancora ulteriori conferme.

Tessuti sede di infiammazione cronica in pazienti con malattie autoimmunitarie quali sclerosi multipla (MS), artrite reumatoide (RA), morbo di Chron (CD) o psoriasi sono caratterizzati da infiltrati di Th17 che producono varie citochine pro infiammatorie quali IL-17, IL-22, IL-26, TNF e linfotossina β.

I Th17 hanno una spiccata capacità di reclutare granulociti ed è quindi verosimile che possano così accentuare l'infiammazione bronchiale in casi di asma complicata da infezioni batteriche. E' invece improbabile una loro implicazione in disordini allergici IgE-mediati perché IL-4 è uno dei più potenti segnali inibitori del differenziamento dei Th17 sia nell'uomo che nel topo.

2. Le cellule presentanti l'antigene (APC)

L'attivazione dei linfociti T richiede che l'antigene venga loro presentato da cellule specializzate dette APC, quali cellule dendritiche (DC), macrofagi e linfociti B. Tali cellule sono in grado di internalizzare, processare e presentare gli antigeni ai linfociti T antigene-specifici inducendone l'attivazione e l'espansione clonale [Delves *et al*., 2000]. Le DC sono efficienti stimolatrici sia dei linfociti B che T. I linfociti B sono in grado di riconoscere anche autonomamente l'antigene nella sua completezza, i linfociti T invece necessitano che questo venga prima processato sotto forma di peptidi e poi presentati dall'APC in associazione ad una molecola del complesso maggiore di istocompatibilità (MHC). Il linfocita è così in grado di riconoscere il frammento antigenico presentato dalla APC (Fig.5).

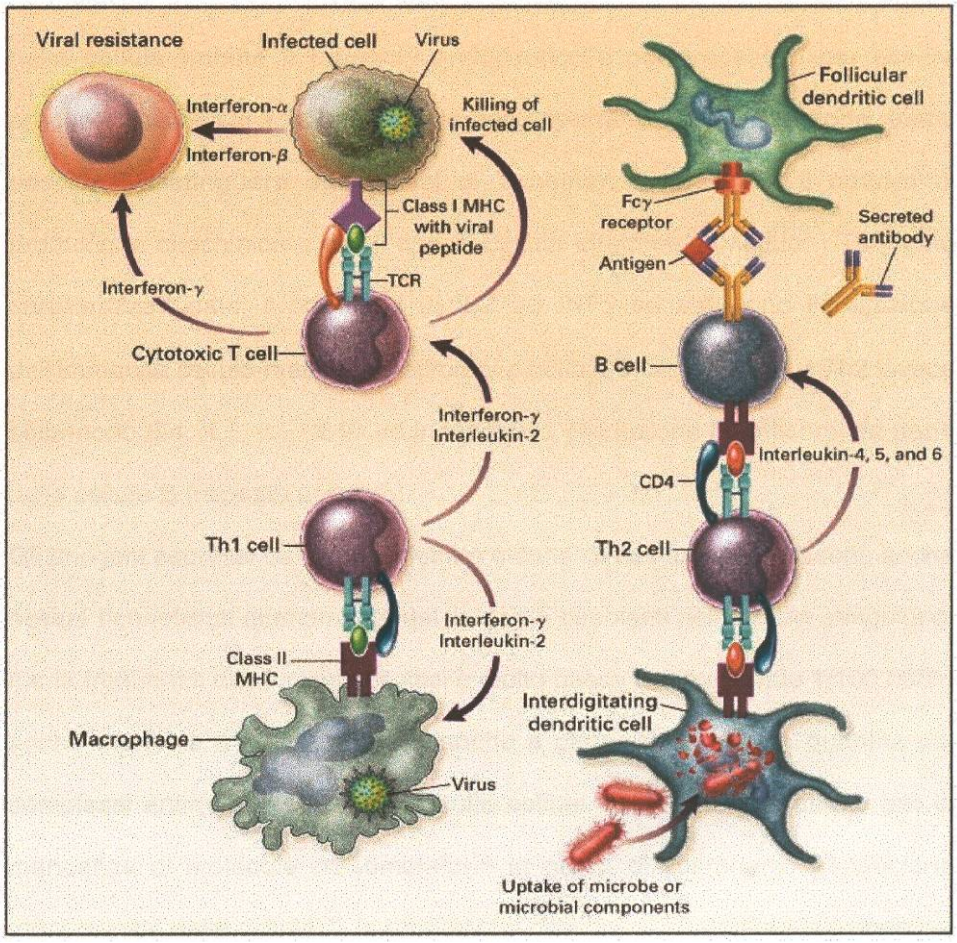

Figura 5 - Schematizzazione delle risposte immunitarie cellulo-mediata ed umorale e dell'interazione delle APC con i linfociti T.

2.1 Le cellule dendritiche

Le cellule dendritiche devono il loro nome alla fitta rete di lunghi processi della membrana che si dipartono dal soma cellulare, simili ai dendriti delle cellule nervose (Fig.6).

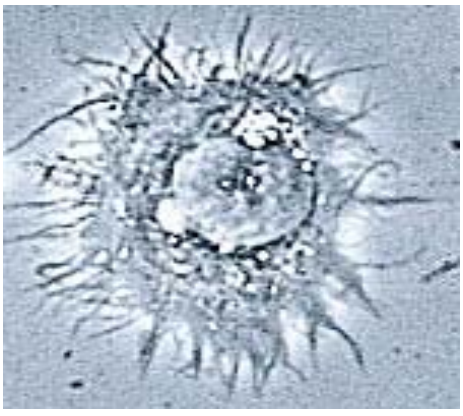

Figura 6 - Morfologia di una cellula dendritica

Derivano dagli stessi precursori dei macrofagi e, durante la maturazione, migrano dal midollo osseo ai tessuti periferici; sono stati identificati vari stadi differenziativi delle DC*:*

progenitori midollari e monociti circolanti che costituiscono la sorgente di DC; *DC immature* residenti nei tessuti e responsabili della captazione e della processazione dell'antigene e infine *DC mature*, situate negli organi linfoidi secondari, dove presentano l'antigene ai linfociti T, attivando la risposta immunitaria specifica [Banchereau J et al., 2000].

A seconda della loro localizzazione possono essere suddivise in:

- Cellule del Langherans nell'epidermide e nelle mucose
- Cellule dendritiche interstiziali
- Cellule dendritiche interdigitate nelle regioni ricche di linfociti T degli organi linfoidi secondari e nel timo
- Cellule dendritiche circolanti nel sangue e nella linfa (cellule velate), distinte in mieloidi e plasmacitoidi.

Sono caratterizzate da elevati livelli di espressione delle molecole MHC sia di classe I che di classe II, oltre ai membri della famiglia B7: per questa ragione sono APC molto più potenti di macrofagi e linfociti B. Oltre alle molecole di classe I e II, le cellule dendritiche esprimono un'altra classe di molecole coinvolte nella presentazione dell'antigene: le proteine della famiglia CD1. E' una famiglia di 5 proteine (CD1a, b, c, d, e) coinvolta nella regolazione delle risposte T contro antigeni microbici lipidici o glicolipidici [Banchereau et al., 2000; Guermonprez et al., 2002].

Le cellule dendritiche rimangono nei tessuti, in forma immatura, fino all'arrivo di un patogeno. Dopo averlo fagocitato si spostano, attraverso il sistema linfatico, al linfonodo più vicino, dove interagiscono con i linfociti *naive*. Se le cellule dendritiche non sono attivate, inducono tolleranza verso l'antigene catturato. Le cellule dendritiche immature presentano sulla loro membrana recettori specifici per componenti comuni di alcuni patogeni: l'interazione recettore-ligando stimola la cellula dendritica a fagocitare il patogeno e a degradarlo a livello intracellulare. Le DC reclutano i leucociti al sito dell'infiammazione e amplificano le risposte immuni innate attraverso la produzione di chemochine, citochine infiammatorie e interferoni di tipo I; l'attività fagocitica diminuisce, mentre aumentano l'espressione di molecole MHC di classe I e II e di molecole costimolatorie CD80 (o B7-1), e CD86 (o B7-2). In seguito ad un riarrangiamento del citoscheletro le DC acquisiscono un'elevata capacità motoria, sostenuta dall'aumentata espressione di recettori per le chemochine. Le cellule dendritiche possono fagocitare anche antigeni extracellulari con meccanismo recettore-indipendente detto macropinocitosi. La principale funzione delle dendritiche non è però quella di distruggere i

patogeni, come invece fanno i macrofagi, ma semplicemente di presentarli ai linfociti T (Fig.7).

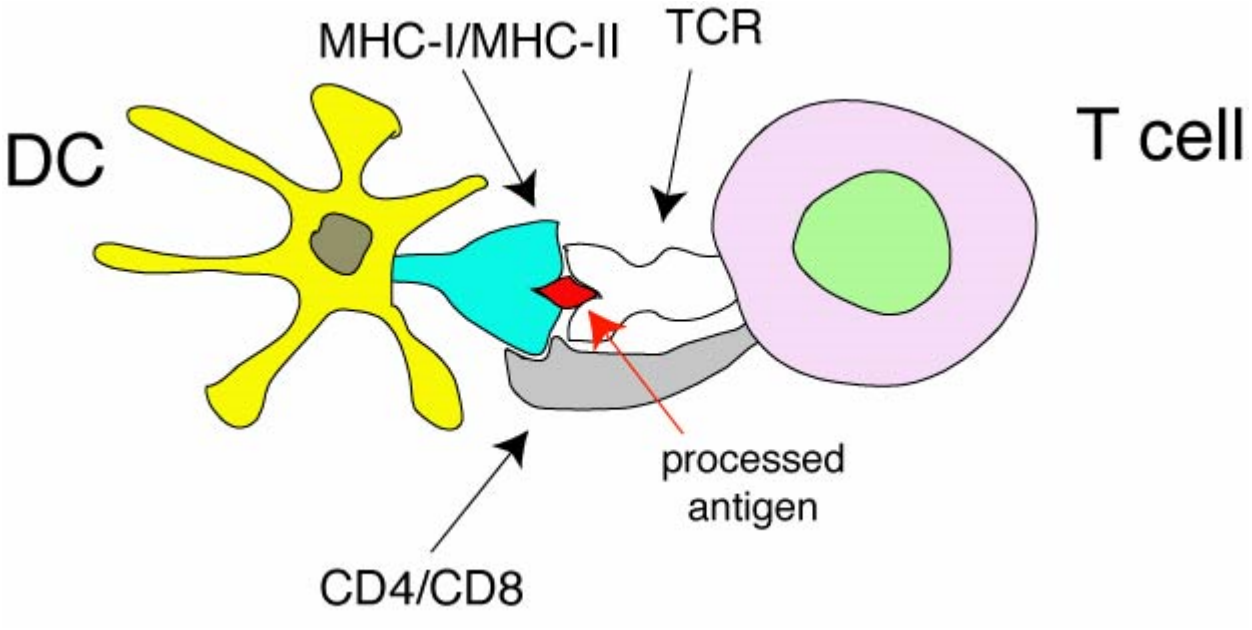

Figura 7 - Interazione tra DC e linfociti T

A loro volta i linfociti T-helper attivano le DC mediante l'interazione CD40-CD40L e le DC così attivate sono capaci di stimolare una risposta T-killer [Bennet SRM et al., 1998; Schoenberger SP et al., 1998]. Le cellule dendritiche sono quindi le cellule più potenti ad indurre una risposta T-linfocitaria ed hanno la peculiare capacità di stimolare non solo le cellule T-memoria, ma anche linfociti T *naïve*.

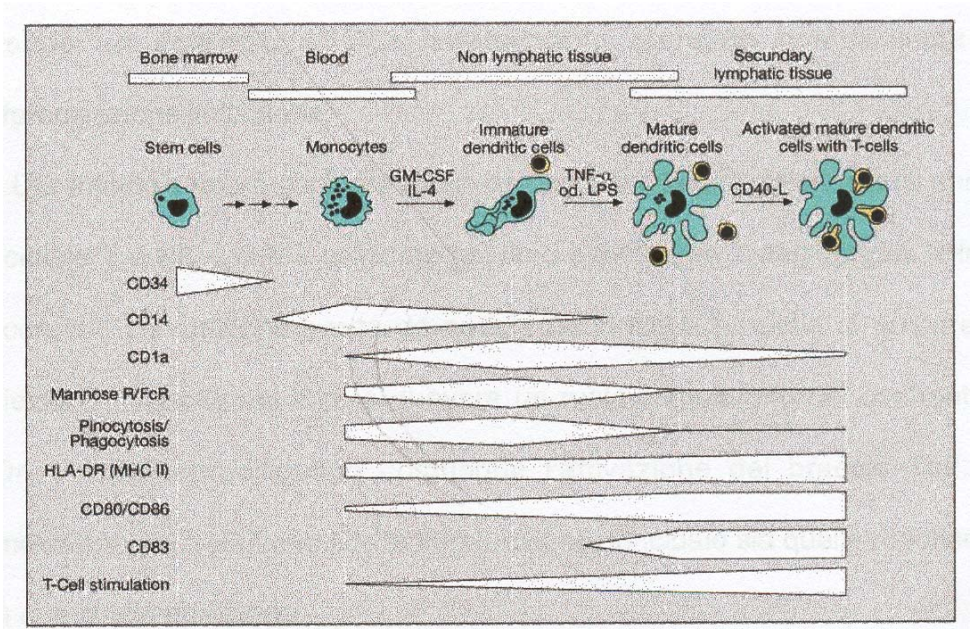

Figura 8 - Maturazione delle cellule dendritiche: modificazione dell'espressione antigenica, dell'attività fagocitica e di quella cross-presentante durante il processo differenziativo.

Le DC stimolano la proliferazione e la produzione di anticorpi da parte dei linfociti B sia indirettamente, grazie all'attivazione dei linfociti T helper, sia direttamente attraverso la produzione di citochine, tra cui l'IL-12 [Palucka et al, 1999]. Le DC inoltre sono in grado di stimolare la citotossicità delle cellule NK sempre con un meccanismo IL-12-dipendente. Le due sottopopolazioni di cellule dendritiche polarizzano in modo diverso il profilo citochinico delle NK: le mieloidi promuovono il rilascio di IFN-γ, mentre le plasmacitoidi stimolano la produzione di IL-4 [Palucka et al., 1999].

2.2 La presentazione dell'antigene

Poiché un antigene proteico estraneo possa essere riconosciuto da un linfocita T, esso deve essere degradato in piccoli peptidi antigenici che si associano fisicamente alle molecole MHC di classe I o di classe II. Inoltre i linfociti, per essere attivati, necessitano di un secondo segnale detto costimolazione. Le APC possiedono sulla loro superficie sia le molecole del complesso MHC sia le molecole costimolatorie e sono quindi in grado di attivare completamente i linfociti T.

2.2.1 Il complesso MHC

Il complesso maggiore di istocompatibilità (MHC) è un grosso complesso genico con loci multipli. Questi codificano due classi principali di glicoproteine di membrana, le molecole MHC di classe I e di classe II. In linea generale i linfociti T_H riconoscono antigeni legati alle molecole MHC di classe II, mentre i linfociti T_C quelli associati a molecole di classe I. Nell'uomo le molecole MHC prendono nome di HLA: sono noti 3 loci genici per la classe I (HLA-A, HLA-B e HLA-C) e 3 loci genici per la classe II (HLA-DR, HLA-DP e HLA-DQ). Ciascuna di queste molecole è caratterizzata da un elevato grado di polimorfismo nella popolazione. Poligenia e polimorfismo del sistema, sono giustificati poiché ciascuna molecola MHC ha una relativa selettività di legame con i peptidi antigenici. Pertanto maggiore è il numero di MHC differenti, maggiore è lo spettro di peptidi antigenici che potranno essere legati e presentati efficacemente ai linfociti T. Quindi ciascun TCR è specializzato nel riconoscimento di un determinato peptide legato ad una determinata molecola MHC, espressa sulla superficie di cellule presentanti l'antigene o di cellule bersaglio. L'espressione delle molecole MHC di classe II è limitata alle APC, mentre quelle di classe I sono espresse da tutte le cellule nucleate.

2.2.2 La famiglia B7

Le molecole B7 sono membri della superfamiglia delle immunoglobuline; possiedono un unico dominio di tipo V e un unico dominio di tipo C.

B7-1 (CD80) e B7-2 (CD86) hanno la stessa organizzazione dei domini extracellulari, ma domini intracellulari molto diversi. B7-2 è costitutivamente espresso a bassi livelli sulle APC ed è rapidamente indotto in seguito all'attivazione. L'espressione di B7-1 invece è indotta in un secondo tempo. Ciò sembrerebbe quindi indicare la maggior importanza di B7-2 nella fase iniziale della risposta immunitaria. Le funzioni di B7-1 e B7-2 sono in gran parte sovrapponibili e sembrano quindi differire principalmente nella cinetica di espressione. I loro ligandi sulla superficie linfocitaria sono CD28 e CTLA4, anch'essi membri della superfamiglia delle immunoglobuline.

Due nuovi membri di questa famiglia sono B7h, ligando di ICOS, e PD-L1/PD-L2, ligandi di PD-1.

2.2.3 B7h, il ligando di H4/ICOS

Il ligando di H4/ICOS è una proteina di 309 aminoacidi espressa sulla superficie delle APC chiamata in vari modi: B7-h, B7RP-1 (B7 related protein-1), LICOS (ligand of ICOS), GL50.

B7h e H4/ICOS interagiscono con una K_D 33 nM e una off-rate con $t_{1/2}$ di 10 minuti.

B7h è costituito da un dominio extracellulare, un dominio transmembrana e una coda citoplasmatica. In particolare possiede un dominio IgV-simile e un dominio IgC-simile analoghi a quelli di B7-1 e B7-2.

A differenza di B7-1 e B7-2, B7-h viene espresso non solo da cellule di derivazione ematopoietica, ma anche da altri tessuti. In particolare è espresso a bassi livelli sui linfociti B, sui macrofagi e sulle cellule dendritiche e aumenta in seguito a stimolazione con IFNγ [Aicher et al., 2000]

3. Il ruolo di ICOS nell'immunità antitumorale

Secondo la teoria dell'immunosorveglianza, il sistema immunitario specifico può riconoscere le cellule tumorali e opporsi allo sviluppo della neoplasia, riconoscendo antigeni tumore-specifici o tumore-associati. Un tumore è in grado di attivare una risposta da parte dei linfociti T se esprime in superficie molecole MHC. Perchè la riposta sia efficiente il linfocita deve però anche ricevere un segnale costimolatorio. Le molecole costimolatorie sono generalmente espresse solo su cellule immunitarie, con l'eccezione di B7h, che e' anche espresso su altri tipi cellulari, tra cui cellule endoteliali vascolari e cellule stromali attivate.

Il bilancio di espressione di molecole costimolatorie e coinibitorie sulla superficie delle cellule tumorali è fondamentale perchè queste possano indurre un'efficace risposta dei linfociti T (*tumor surveillance*). La mancata espressione di molecole costimolatorie rende il tumore invisibile al sistema immunitario, così come una aumentata espressione di molecole inibitorie. Questo modello è stato confermato in modelli murini, in cui è stato studiato l'effetto dell'espressione ectopica di B7.1 e B7.2 sulle cellule tumorali [Townsend S.E., 1993; Chen L., 1992; Baskar S., 1993]. L'induzione dell'espressione di B7.1 su cellule tumorali mediante trasfezione è sufficiente a indurre rigetto mediato da linfociti T CD8+ e CD4+. Il rigetto inoltre genera memoria immunologica e una conseguente immunità alla successiva induzione dello stesso tipo di tumore. Lo stesso approccio è stato usato per dimostrare che l'espressione ectopica di B7h è in grado di fornire il necessario segnale costimolatorio e di promuovere il rigetto CD8-mediato di fibrosarcoma e plasmacitoma [Wallin JJ, 2001; Liu X, 2001]. L'ottimale espansione clonale dei linfociti T CD8+ indotta da B7h richiede però la presenza di B7.1 e B7.2 sulle APC dell'ospite (Fig.9).

Sono già in corso *trials* clinici per testare l'efficacia di vaccini basati su cellule tumorali trasfettate con B7.1:

- carcinoma renale metastatico: cellule tumorali autologhe trasfettate con B7.1 in associazione a somministrazione sistemica di IL-2 [Antonia SJ, 2002]
- carcinoma polmonare a cellule "non-piccole": linea di adenocarcinoma trasfettata con B7.1 e HLA-A1 o A2 [Raez LE, 2004].
- leucemia mieloide acuta: cellule tumorali autologhe trasfettate con B7.1 in associazione a IL-2 e trapianto di cellule staminali ematopoietiche allogeniche [Chan L, 2006].

Nell'analizzare i precedenti studi bisogna tenere conto del fatto che B7.1 lega non solo CD28, che è notoriamente costimolatrice, ma anche CTLA4, il prototipo dei recettori inibitori. E' quindi possibile, in certe circostanze, che questi vaccini possano attenuare piuttosto che potenziare la risposta immunitaria. Infatti l'espressione di bassi livelli di B7.1 è uno dei meccanismi di immunoelusione riscontrati in modelli murini di carcinoma del colon [Tirapu I, 2006].

Anche il blocco di CTLA4 con anticorpi neutralizzanti ha dimostrato benefici clinici in pazienti con cancro.

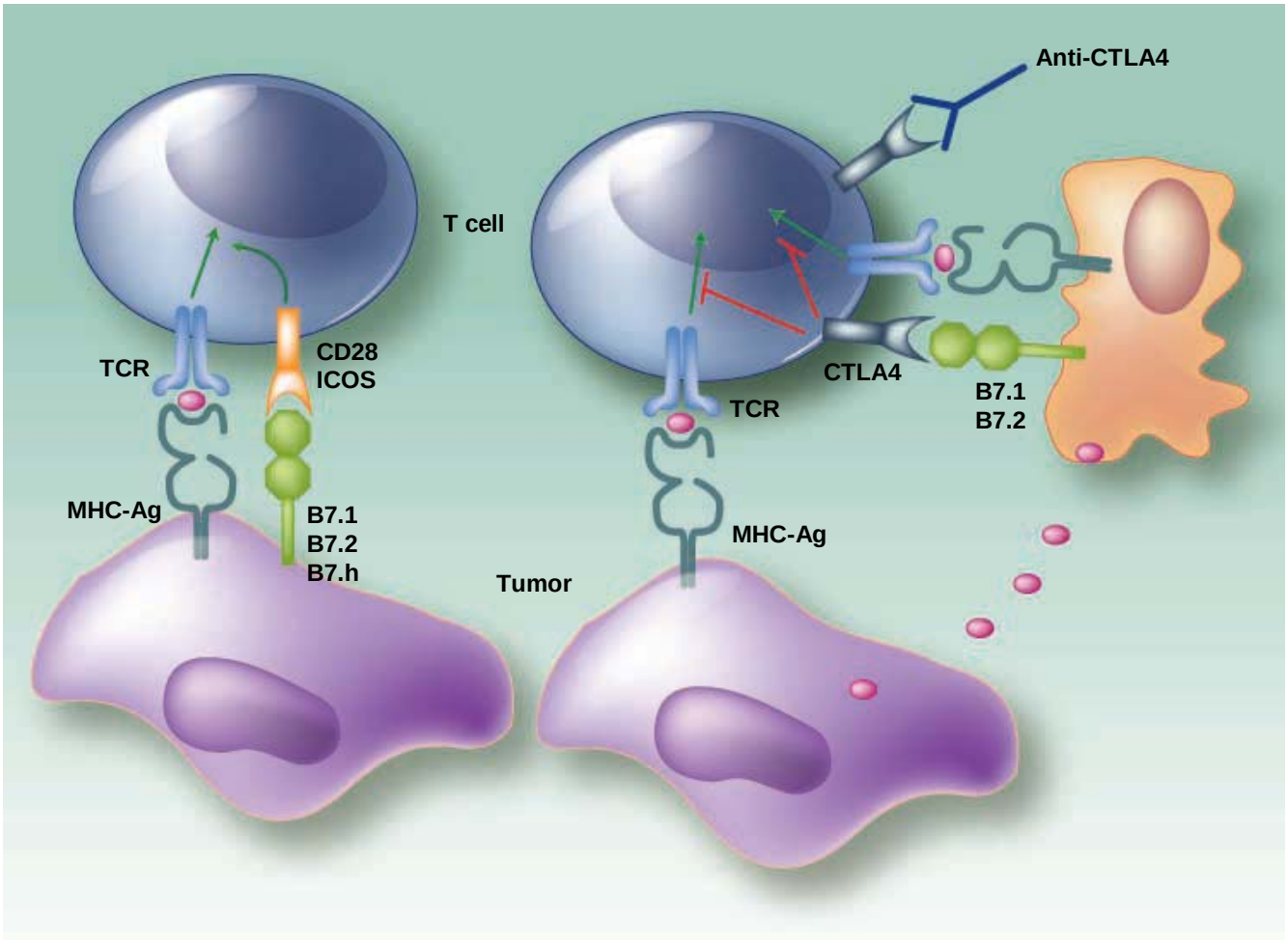

Figura 9 – [Clin Cancer Res 2007;13(18) September 15, 2007]

Tutti gli studi finora realizzati hanno usato come modello tumori trapiantabili o indotti chimicamente, mentre mancano dati sul ruolo di ICOS/B7h in modelli di tumore spontaneo con stadi di sviluppo molto più simili al corrispettivo umano, come quello rappresentato dal topo BALB-neuT.

4. Immunobiologia del ceppo murino BALB-neuT

La morfogenesi delle ghiandole mammarie è controllata dagli ormoni sessuali, ovvero estrogeni e progestinici, e da fattori di crescita autocrini. Alterazioni nei segnali di crescita influenzano sia il differenziamento che la proliferazione dei componenti strutturali di tali ghiandole e possono essere associate allo sviluppo di neoplasia e iperplasia.

Tali alterazioni possono per esempio essere dovute a cambiamenti nel numero e nella funzione dei recettori dei suddetti fattori di crescita. Tra i recettori di fattori di crescita espressi sulla membrana delle cellule mammarie si trova p185neu, una proteina con importante funzione morfogenetica e la cui alterazione è spesso di importanza critica nella trasformazione neoplastica.

p185neu è un recettore di membrana ad attività tirosinchinasica, codificato dall'oncogene ErbB-2. E' costituito da una regione extracellulare contenente 4 domini associati a 2 a 2 per formare unità a 2 domini, da un singolo dominio transmembrana e da un dominio ad attività tirosinchinasica intracitoplasmatico.

L'analogo di tale oncogene nei roditori è chiamato Her-2/neu, poichè fu per la prima volta identificato in cellule di neuroblastoma.

4.1 La proteina p185neu

p185neu appartiene alla famiglia degli *Epidermal Grawth Factors* (EGF). I recettori di questa famiglia sono in grado di legarsi ad un ampio gruppo di molecole, delle quali EGF è il prototipo. A questa famiglia appartengono anche la proteina EGFR/ErbB-1 e i prodotti degli oncogeni ErbB-3 ed ErbB-4. Non è stato finora scoperto un ligando specifico per p185 ed è probabile che non esista: infatti p185neu ha una struttura aperta in grado di funzionare come corecettore di tutti gli altri ErbB, anche in assenza di interazione diretta con un ligando. Molti membri della famiglia ErbB, una volta associati al loro ligando, formano eterodimeri con altri recettori di EGF e preferibilmente con p185neu, e attivano così una cascata intracellulare di trasduzione del segnale dipendente da MAPK e PI3K/AKT, con effetti potentemente mitogenici e anti-apoptotici.

E' stato ipotizzato che la sovraespressione di p185neu promuova la formazione di nuovi eterodimeri, aumentando così l'effetto mitogenico.

I recettori della famiglia di EGFR (Fig.10) e in particolar modo p185 sono determinanti per morfogenesi e trofismo di molti tessuti: sono infatti coinvolti nel differenziamento delle cellule mesenchimali ed epiteliali, nello sviluppo di neuroni e cellule gliali, del cuore e delle ghiandole mammarie. Un loro malfunzionamento e' spesso associato a patogenesi e

progressione di tumori, soprattutto mammari. Infatti tumori mammari con amplificazione di ErbB-2 e sovraespressione di p185neu dimostrano maggior aggressività e invasività oltre ad una maggior resistenza sia alla chemioterapia che alla terapia ormonale.
Non solo queste caratteristiche rendono p185neu un target ideale in trattamenti sperimentali, ma anche il fatto che la percentuale di cellule p185neu+ è generalmente maggiore nelle lesioni preneoplastiche, nei tumori primari e nelle metastasi.

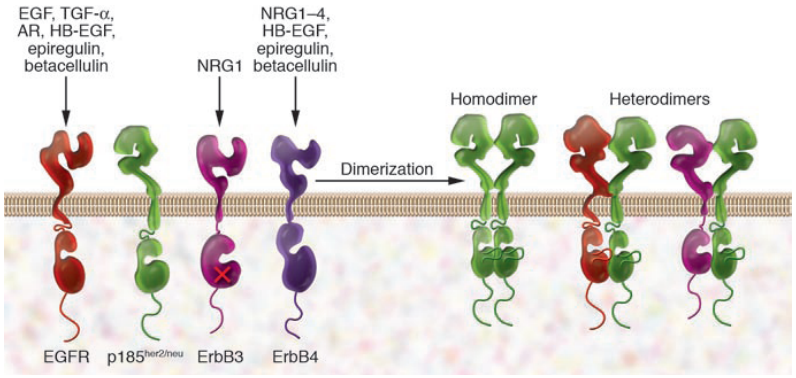

Fig 10 - I recettori della famiglia di EGFR [Pannellini et al., 2004]

4.2 Vaccinazione verso p185^{neu}

p185neu non e' una molecola particolarmente immunogenica perchè è un antigene *self*, espresso in molti tessuti sani e semplicemente sovraespresso in cellule tumorali. In certi casi però questa sovraespressione è in grado di sovvertire la tolleranza immunologica: si è infatti riscontrato che alcuni pazienti producono Th, CTL e anticorpi specifici per p185neu.
Molti vaccini sovvertono la tolleranza a p185neu e inducono una risposta umorale e cellulare in pazienti affetti da tumori con alterata funzione di ErbB-2.
p185neu può essere considerato il prototipo di una classe di *Tumor-Associated Antigens* (TAA) con la caratteristica, molto utile a fini terapeutici e diagnostici, di essere espressi già in lesioni preneoplastiche e di incrementare la loro espressione con la progressione del tumore.

4.3 Topi BALB/c transgenici per l'oncogene Her2/neu

Un modo per testare il potenziale del sistema immunitario nella prevenzione e cura dei tumori è quello di utilizzare topi geneticamente modificati, cioè in grado di sviluppare tumore come conseguenza della mutazione o sovraespressione di un particolare oncogene oppure della perdita di funzione di un oncosoppressore.

In tali modelli murini i tumori diventano evidenti dopo un periodo di tempo caratterizzato dai progressivi stadi della tumorigenesi, mentre la relazione tra tumore e tessuti circostanti è conservata. Altre importanti caratteristiche di questi modelli, che concorrono a confermare una stretta analogia con tumori umani, sono il carattere autologo dei tumori, lo sviluppo di metastasi e la presenza di un sistema immunitario intatto.
Ci sono però anche differenze rispetto al corrispettivo umano, di cui tenere in conto: per esempio il *pattern* di espressione potrebbe non corrispondere a causa della specificità del promotore genico e i meccanismi patogenici possono non sovrapporsi completamente.
Anche il momento in cui avviene la prima espressione dell'oncogene è di cruciale importanza immunologica perchè può direttamente influenzare l'intensità della tolleranza.
Alcuni modelli inoltre sono difficilmente gestibili perchè il tumore ha un lungo periodo di latenza o si sviluppa solo in una piccola percentuale di topi o solo in particolari condizioni come la gravidanza o in seguito alla mutazione di geni che non hanno un corrispettivo umano. L'utilizzo di modelli tumorali murini richiede quindi un compromesso.
Sono stati sviluppati e descritti circa un centinaio di modelli murini di tumore mammario spontaneo: ci sono diverse linee di topi transgenici che cominciano a sovraesprimere Her2/neu di ratto in differenti momenti della vita [Muller et al., 1988]. La trascrizione di questo oncogene è controllata da una sequenza ripetuta di *mammary tumor virus*, che conferisce rapidità di sviluppo e accentuata aggressività, oltre a localizzare il tumore stesso a livello delle ghiandole mammarie. La mutazione puntiforme in posizione 664 nel dominio transmembrana porta alla sostituzione di una Valina con un Acido Glutammico, la cui carica negativa risulta nella formazione di legami a idrogeno con un'Alanina in posizione 661 di una seconda molecola di p185neu o di altri recettori EGF. Questi omo- ed eterodimeri trasducono spontaneamente segnali proliferativi, anche in assenza di ligando.
Uno dei più aggressivi modelli di carcinogenesi mammaria dovuta a rat Her-2/neu è quello su background di femmine BALB/c: lo *strain* derivante si chiama BALB-neuT ed è stato ottenuto da un topo transgenico Her2/neu *non-inbred* dopo circa 3 anni di incroci con BALB/c.

4.4 Morfologia dello sviluppo del tumore mammario in topi BALB-neuT

Alla nascita, le 10 ghiandole mammarie murine dei topi BALB-neuT consistono in un singolo dotto galattoforo che si suddivide in 3-5 dotti secondari. Dalla quarta settimana questi iniziano a suddividersi per formare nuovi dotti. Essi terminano in piccoli sacchi rivestiti da parecchi strati di cellule epiteliali (*terminal end buds)*: proprio questi sono la

zona più sensibile a stimoli proliferativi e proseguono il loro sviluppo estrogeno-dipendente fino alla ottava-dodicesima settimana. Allo stesso tempo cominciano a formarsi ulteriori ramificazioni duttali, duttuli e alveoli e i cosiddetti *lateral buds*, che sono la zona di maggior espressione di rat p185neu e il *focus* iperplasico durante la carcinogenesi. All'ottava settimana nelle sacche laterali cominciano ad apparire carcinomi *in situ* e a moltiplicarsi i *foci* di iperplasia (Fig.11) .

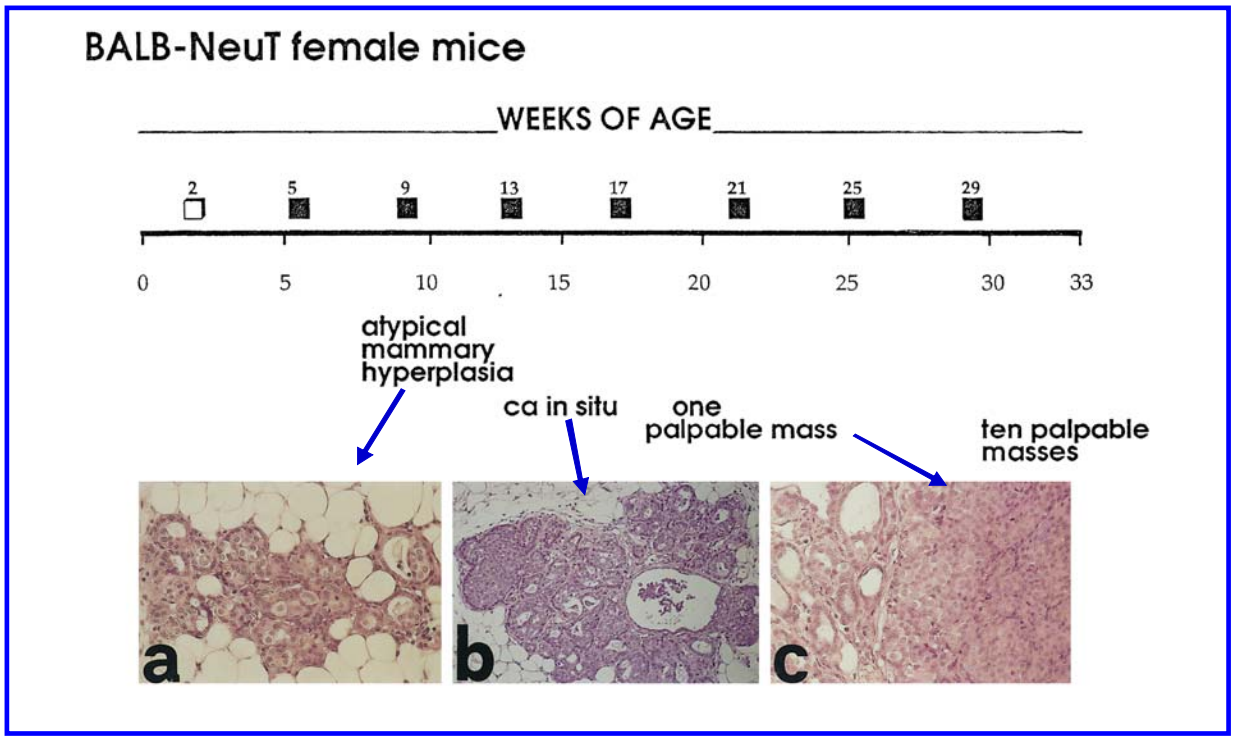

Fig.11 – Cinetica di sviluppo del tumore mammario in BALB-neuT [da Pannellini et al., 2004]

Questo processo avviene in parallelo in tutte le 10 ghiandole mammarie. Il carcinoma *in situ* diventa invasivo tra la decima e la ventesima settimana e metastatizza dopo la 35-sima. Già durante le prime fasi di sviluppo del tumore, nel modello BALB-neuT, si attiva l'angiogenesi.

Il modello BALB-neuT è particolarmente utile per lo studio e il perfezionamento di strategie immunoterapiche volte a inibire lo sviluppo di tumori. Il recettore p185 neu è un ottimo bersaglio immunologico, essendo una proteina di membrana facilmente accessibile anche senza presentazone in associazione ad MHC, la cui espressione è spesso alterata in tumori unani e murini.

4.5 Vaccinazione antitumorale in topi BALB-neuT

Basandosi sulle caratteristiche morfologiche e funzionali di tale modello, sono stati disegnati vaccini, sia cellulari che a DNA, coadiuvati da molecole ad effetto anti-angiogenetico ed immunostimolante.

a – Vaccinazione cellulare

Consiste nella somministrazione/trasferimento di cellule tumorali mammarie allogeniche esprimenti rat p185neu e molecole H-2^q di classe I (cellule neu/$H2^q$) a partire dalla sesta settimana (quando sono presenti solo *foci* iperplastici), seguita dalla somministrazione sistemica di IL-12, ad effetto prevalentemente antiangiogenetico. Questo trattamento è in grado di prevenire lo sviluppo di carcinomi invasivi in tutti i topi di 22 settimane e l'88% rimane libero da tumori palpabili fino alla 52-sima settimana. L'effetto dei due componenti è additivo/sinergico. I linfociti CD8+ prelevati dalla milza di topi trattati producono elevate quantità di IFN-γ.

b – Vaccinazione a DNA

La vaccinazione cellulare comporta vari rischi e problemi tecnici. Per questo motivo si è pensato di vaccinare con segmenti di DNA (plasmidi) codificanti per rat p185neu o una sua porzione. La somministrazione di tali plasmidi, da soli o in combinazione a segmenti codificanti per la molecola immunomodulatoria IL-1β è in grado di ritardare sensibilmente la comparsa dei tumori e di diminuirne la molteplicità. Questo effetto protettivo però declina col tempo e alla settimana 52 quasi nessun topo è libero da tumore.
Quindi l'obiettivo più importante non è tanto quello di aumentare semplicemente la risposta immunitaria ma quello di ottenere un prolungamento della memoria immunlogica.

Le vaccinazioni cellulare e a DNA sono in grado di prevenire la progressione solo di lesioni tumorali precoci, ma diventano molto meno efficienti negli stadi successivi. Inoltre sono totalmente inefficaci se iniziate dopo la settimana 15 di vita, quando sono già presenti lesioni avanzate. Sia nell'uomo che nel topo i tumori possono evadere la sorveglianza immunologica attraverso vari meccanismi come la produzione di molecole ad attività immunosoppressiva o la creazione di un microambiente fisicamente inaccessibile al sistema immunitario stesso. Inoltre esistono cloni tumorali che non esprimono l'antigene-target del vaccino e la distuzione, in seguito alla vaccinazione, dei cloni positivi può favorire l'espansione di tali cloni resistenti. Migliori risultati sono stati ottenuti con l'immunoterapia preventiva e cioè vaccinando prima della comparsa dei tumori [Pannellini et al., 2004]

Scopo del lavoro

La molecola H4/ICOS, espressa sulla superficie dei linfociti T attivati, svolge un ruolo chiave nella modulazione della risposta immunitaria interagendo con il suo ligando B7h, costitutivamente espresso dalle cellule presentanti l'antigene professioniste (APC), ovvero cellule dendritiche (DC), macrofagi e linfociti B, ma anche da altri tipi cellulari.

L'obiettivo del mio lavoro è stato studiare il ruolo dell'interazione di ICOS e B7h nella risposta immunitaria per prima cosa valutando gli effetti della stimolazione di ICOS in linfociti *naïve* umani. E' infatti ben noto il ruolo di ICOS nella risposta secondaria di cellule già attivate ma poco si sa delle sue funzioni in cellule vergini.

Quindi ho voluto studiare gli effetti di questa coppia di recettori non solo sui linfociti ma anche sulle altre cellule interessate, le APC. Anche questi studi sono stati effettuati su cellule umane. Si parla in questo caso di *reverse signaling.* Dopo uno *screening* iniziale su DC, macrofagi e linfociti B abbiamo deciso di approfondire lo studio delle DC, che hanno dato i risultati più interessanti. I risultati ottenuti ci hanno portato a ipotizzare un ruolo di ICOS nel differenziamento dei Th17.

Alla luce dei risultati ottenuti e basandoci sulla letteratura, abbiamo voluto investigare il possibile ruolo dell'interazione ICOS-B7h nella risposta immunitaria anti-tumorale. Abbiamo scelto un modello murino di carcinoma mammario e in particolare il ceppo BALB-neuT perché ad oggi non si ritrovano in letteratura studi relativi a tale coppia recettoriale in tumori spontanei.

Risultati (1)

In letteratura prevale l'opinione che ICOS sia coinvolto soprattutto nella modulazione della funzione delle cellule T precedentemente attivate e la maggior parte dei dati disponibili sono stati ottenuti nel modello murino. Pertanto questo lavoro si è proposto di valutare gli effetti di ICOS in linfociti T CD4+ *naïve* umani.

Per fare ciò sono state utilizzate le forme solubili dei ligandi fisologici di CD28 (quale controllo) e ICOS, ovvero rispettivamente B7.1-Ig e GL50-Ig, in un modello di attivazione *in vitro* indotta da anticorpi anti-CD3. Gli esperimenti condotti hanno dimostrato che la stimolazione di ICOS funziona da *switching factor* fondamentale per permettere l'attivazione di linfociti stimolati da dosi sub-ottimali di CD3+CD28 oppure dal solo CD3 in presenza di IL-2 esogena, potenziando in modo significativo la secrezione di IL-2, IFN-γ, IL-10 e TNF-α, ma non di IL-4. Utilizzando anticorpi antagonisti per IL2 e IFN- γ, abbiamo dimostrato che la stimolazione di ICOS stabilisce un *feedback* positivo con IFN-γ, che richiede la presenza di IL-2 ed è inibito da IL-4. Viceversa in assenza di stimolazione di CD28 o di IL-2 esogena, la stimolazione di ICOS induce l'espressione di FoxP3 e TGF-β1 e il differenziamento di linfociti T regolatori in grado di inibire la proliferazione di linfociti T CD4+ indotta da cellule allogeniche.

I nostri risultati supportano quindi la tesi che ICOS è in grado di pilotare il differenziamento verso dei linfociti T helper effettori (prevalentemente di tipo T_H1) se associato a costimoli appropriati quali CD3+CD28 o CD3+IL-2 e verso linfociti T regolatori in assenza di questi costimoli.

E' degno di nota, a questo proposito, che B7h sia costitutivamente espresso a livelli elevati su molti tipi di cellule della linea emopoietica e non, mentre B7.1 e B7.2 siano espressi solo dalle APC attivate. Questo suggerisce che quando è assente la stimolazione di CD28 (ovvero in assenza di APC attivate) ICOS inibisce l'attivazione della risposta immunitaria favorendo la differenziazione di Treg. D'altra parte ICOS potrebbe svolgere un ruolo chiave anche nell'induzione di una risposta T_H1 in situazioni in cui nel microambiente siano presenti alti livelli di IL-2 ma non APC attivate. Pertanto ICOS potrebbe avere un ruolo chiave nell'induzione della risposta immunitaria contro antigeni deboli nell'ambito di una risposta contro altri antigeni più potenti, il che potrebbe risultare importante nello sviluppo di malattie autoimmuni. In linea con questa possibilità, vari modelli murini di malattie autoimmuni sono stati associati a una aumentata espressione di ICOS.

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ICOS cooperates with CD28, IL-2, and IFN-γ and modulates activation of human naïve CD4$^+$ T cells

Riccardo Mesturini[1], Stefania Nicola[1], Annalisa Chiocchetti[1], Ilaria Seren Bernardone[1], Luca Castelli[1], Thea Bensi[1], Massimo Ferretti[1], Cristoforo Comi[1], Chen Dong[2], Josè Maria Rojo[3], Junji Yagi[4] and Umberto Dianzani[1]

[1] Interdisciplinary Research Center of Autoimmune Diseases and Department of Medical Sciences, "A. Avogadro" University of Eastern Piedmont at Novara, Novara, Italy
[2] Department of Immunology, University of Texas MD Anderson Cancer Center, Houston, TX, USA
[3] Centro de Investigaciones Biológicas, CSIC, Madrid, Spain
[4] Department of Microbiology and Immunology, Tokyo Women's Medical University, Tokyo, Japan

Several sets of data indicate that ICOS regulates cytokine production in activated T cells, but is less effective on naïve T cells. This work evaluates ICOS function in human naïve CD4$^+$ T cells through an assessment of the effect of soluble forms of the ICOS and CD28 physiological ligands on activation driven by anti-CD3 mAb. ICOS strikingly potentiated secretion of IL-2, IFN-γ, IL-10, and TNF-α, but not IL-4, promoted by optimal stimulation of CD3+CD28, and it was the key switching-factor of activation when cells received suboptimal stimulation of CD3+CD28 or stimulation of CD3 alone in the presence of exogenous IL-2. In these conditions, blockade of IL-2 and IFN-γ showed that ICOS builds up a positive feedback loop with IFN-γ, which required IL-2 and was inhibited by IL-4. By contrast, in the absence of CD28 triggering or exogenous IL-2, ICOS-induced costimulation mainly supported expression of TGF-β1 and FoxP3 and differentiation of regulatory T cells capable to inhibit proliferation of naïve CD4$^+$ T cells driven by allogeneic cells. These data suggest that ICOS favors differentiation of Th effector cells when cooperates with appropriate activation stimuli such as CD3+CD28 or CD3+IL-2, whereas it supports differentiation of regulatory T cells when costimulatory signals are insufficient.

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Introduction

Naïve T cell are fully activated when they receive at least two signals from APC. The first signal is delivered through the TCR/CD3 complex after recognition of the antigenic peptide presented by the appropriate MHC molecule, the second is provided by T cell costimulatory molecules engaged by their ligands expressed on APC. In the absence of costimulatory signals, T cells become anergic or die due to apoptosis.

The best known costimulatory molecule is CD28, which is constitutively expressed by T cells and interacts with B7.1 (CD80) and B7.2 (CD86) on APC; it cooperates with signals delivered by the TCR complex to favor T cell proliferation and cytokine secretion and inhibits induction of anergy and apoptosis [1].

Correspondence: Umberto Dianzani, Department of Medical Science, University "A. Avogadro" of Eastern Piedmont, via Solaroli 17, 28100 Novara, Italy
Fax: +39-0321-620421
e-mail: dianzani@med.unipmn.it.
Abbreviations: iDC: immature DC · **FoxP3:** Forkhead Box P3

Two other molecules structurally similar to CD28 have been assigned to its family, namely CTLA-4 and ICOS. Like CD28 itself, they are selectively expressed by T cells, but their expression depends on cell activation [2].

CTLA-4 binds the same ligands as CD28, *i.e.*, B7.1 and B7.2, but with higher affinity, and delivers negative signals involved in switching-off activated T cells, as a consequence of both direct inhibition of TCR signaling and competition with CD28 binding to B7.1 and B7.2 [3].

ICOS binds a distinct ligand belonging to the B7 family, *i.e.*, B7h (or B7H2, B7RP1, GL50, LICOS), expressed by cells of both hematopoietic and non-hematopoietic origin; like CD28, it costimulates T cell proliferation and secretion of several cytokines. It mainly regulates cytokine production in recently activated T cells, and is less effective in primary activation of naïve T cells, whose activation seems to be strictly dependent on CD28-mediated costimulation [4–11].

Several findings support a functional relationship between ICOS and Th2 development. In the first place, Th2 cells express higher levels of ICOS than Th1 cells [12–14]. Furthermore, blockade or absence of ICOS/B7h interactions favor Th1 differentiation *in vitro* [15–18]. In ICOS-deficient mice, defective ICOS-mediated costimulation exacerbates development of experimental autoimmune encephalomyelitis (EAE), which is a Th1-mediated autoimmune disease, and decreases immune responses to *N. brasiliensis* and lung allergic reactions to protein antigens, which are predominantly Th2-mediated [16, 19–21]. However, there is also evidence that ICOS contributes to Th1 responses in some circumstances [5, 22]. For instance, ICOS-deficient mice display resistance to collagen-induced arthritis [23]. Moreover, *in vivo* blockade of the ICOS/B7h interaction increases allograft survival or accelerates rejection in different experimental settings [24, 25]. ICOS function in $CD8^+$ cells is less known. Its involvement in the anti-tumor response and the response to intracytoplasmic pathogens [20, 26–29], as well as in the differentiation of regulatory T cells (Treg), which produce high levels of IL-10, has been suggested [21, 30].

Most of these data have been obtained in the mouse. The few data from humans point to a different behavior. For instance, our work in the mouse suggests that IL-4 and ICOS build up a positive feedback loop, in which initial secretion of small amounts of IL-4 induces expression of ICOS, whose triggering then enhances IL-4 secretion [31, 32], whereas in humans ICOS expression appears to be up-regulated by IL-12 and IL-23, and is higher in Th1 than in Th2 cells, and ICOS itself appears to play no part in Th1:Th2 polarization [33, 34].

The present paper provides a clearer picture of ICOS function in the primary immune response in humans drawn from a comparison of the effect of CD28 and ICOS triggering by soluble forms of their physiological ligands (*i.e.*, B7.1-Ig and GL50-Ig, respectively) on activation of purified naïve $CD4^+$ T cells. Results show that ICOS favors differentiation of Th effector cells when cooperating with appropriate activation stimuli such as CD3+CD28 or CD3+IL-2, whereas it predominantly supports differentiation of Treg when costimulatory signals are insufficient.

Results

Synergy of ICOS- and CD28-induced costimulation

To evaluate the effect of ICOS-mediated costimulation on naïve $CD4^+$ T cells, we compared the effect induced by costimulation of CD28, ICOS, or both triggered by B7.1-Ig (4 μg/mL) and GL50-Ig (5 μg/mL) on cell activation induced by 0.1 μg/mL ($CD3^{low}$), 1 μg/mL ($CD3^{int}$) or 10 μg/mL ($CD3^{high}$) of anti-CD3 mAb. Secretion of cytokines (IL-2, IL-4, IL-5, IL-10, IFN-γ, and TNF-α), expression of ICOS and CD25, and T cell proliferation were evaluated at day 3 of culture. Results showed that optimal stimulation of CD3+CD28 (using $CD3^{int}$) induced proliferation and expression of both CD25 and ICOS (Fig. 1A), and secretion of IL-2, IFN-γ, and TNF-α, and low levels of IL-5 and IL-10 (Fig. 1B), whereas IL-4 was undetectable (data not shown). The $CD3^{int}$+CD28+ICOS stimulation further increased secretion of IL-10 (fivefold), IL-2 (at least fivefold), IFN-γ (fourfold), TNF-α (twofold), and IL-5 (twofold), whereas IL-4 was still undetectable; moreover, it further increased cell proliferation and surface expression of ICOS and CD25. By contrast, the $CD3^{int}$ or $CD3^{int}$+ICOS stimulation did not induce substantial cell proliferation and cytokine secretion, whereas expression of CD25 and ICOS were weakly induced by CD3+ICOS. As described below, stimulation of CD3+ICOS promoted a weak cell activation in the absence of CD28 triggering only when high doses of anti-CD3 mAb ($CD3^{high}$) were used.

Since B7.1 is a ligand of both CD28 and CTLA-4, we performed the same experiments in the presence of Ab blocking CTLA-4 to assess the influence of this receptor in these experimental conditions. Results showed that blocking of CTLA-4 did not influence the effect of the CD28-induced costimulation either alone or combined with ICOS triggering. Moreover, CTLA-4 expression was undetectable in unstimulated naïve T cells and only a weak expression was detectable at day 3 of culture (data not shown).

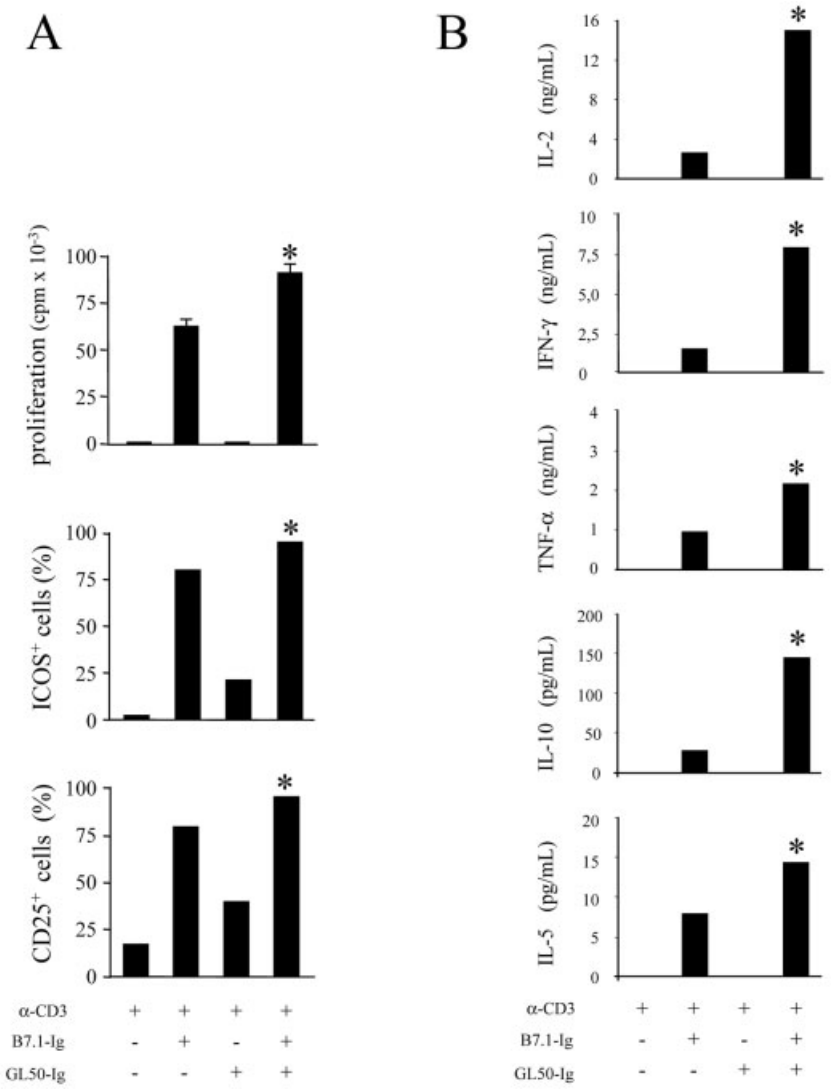

Figure 1. ICOS costimulation of naïve CD4$^+$ T cells activated *via* CD3 and CD28. Naïve CD4$^+$ T cells were stimulated by immobilized anti-CD3 (CD3^{int}: 1 μg/mL) in the presence or absence of GL50-Ig (5 μg/mL), B7.1-Ig (4 μg/mL), or GL50-Ig + B7.1-Ig. (A) The uptake of [^{3}H]thymidine during the last 6 h of 3-day culture is expressed in cpm; ICOS and CD25 expressions were evaluated on day 3 of culture and are shown as percentage of positive cells. (B) Culture supernatants were collected after 3 days of culture for the cytokine assay. Cytokine secretion evaluated at day 2 of cultures detected lower concentrations, but the same trend (data not shown). Results are from one of four representative experiments. Error bars indicate the SD from triplicate cultures in the proliferation assays. Cytokine analysis was performed on mixed supernatants from triplicate cultures. * Significant differences obtained using GL50+B7.1 *vs.* B7.1 ($p<0.05$, Mann-Whitney test for paired samples calculated on the four experiments).

These data suggest that ICOS contributes to primary activation of human naïve Th cells by working in synergy with CD28.

Role of IFN-γ and IL-2 in ICOS-induced costimulation

The highest effect of ICOS-induced costimulation was detected on the pro-inflammatory cytokines IL-2 and IFN-γ and the anti-inflammatory cytokine IL-10. To dissect the reciprocal interference of these cytokines, we used anti-IL-2 and -IFN-γ mAb to neutralize these cytokines.

To better detect ICOS-mediated costimulation, these experiments were performed with optimal amounts of GL50-Ig and B7.1-Ig and suboptimal amounts of anti-CD3 mAb (CD3^{low}). The CD3^{low}+CD28 stimulation induced no cell proliferation (Fig. 2A), low ICOS expression (Fig. 2B), and undetectable cytokine secretion (Fig. 2C).

By contrast, association of these stimuli with ICOS triggering brought all three parameters to levels comparable to those obtained with the optimal CD3^{int}+CD28 stimulation. ICOS-induced costimulation is thus an essential third signal for T cell activation in these experimental conditions.

Blockage of either IL-2 or IFN-γ strikingly inhibited cell proliferation (Fig. 2A) and ICOS expression (Fig. 2B); moreover, anti-IL-2 inhibited secretion of all cytokines, whereas anti-IFN-γ inhibited secretion of TNF-α and IL-2 and increased secretion of IL-5 and IL-10 (Fig. 2C).

In mouse naïve Th cells, IL-4 builds up a positive feedback loop with ICOS, in which IL-4 induces ICOS expression whose triggering enhances IL-4 secretion. This loop seems not to work in human cells since IL-4 was not substantially induced in any culture condition (data not shown). However, we directly assessed the effect of IL-4 on ICOS expression by adding 2 ng/mL IL-4 to lymphocytes activated using the CD3^{low}+CD28 stimulus in the presence and absence of ICOS triggering.

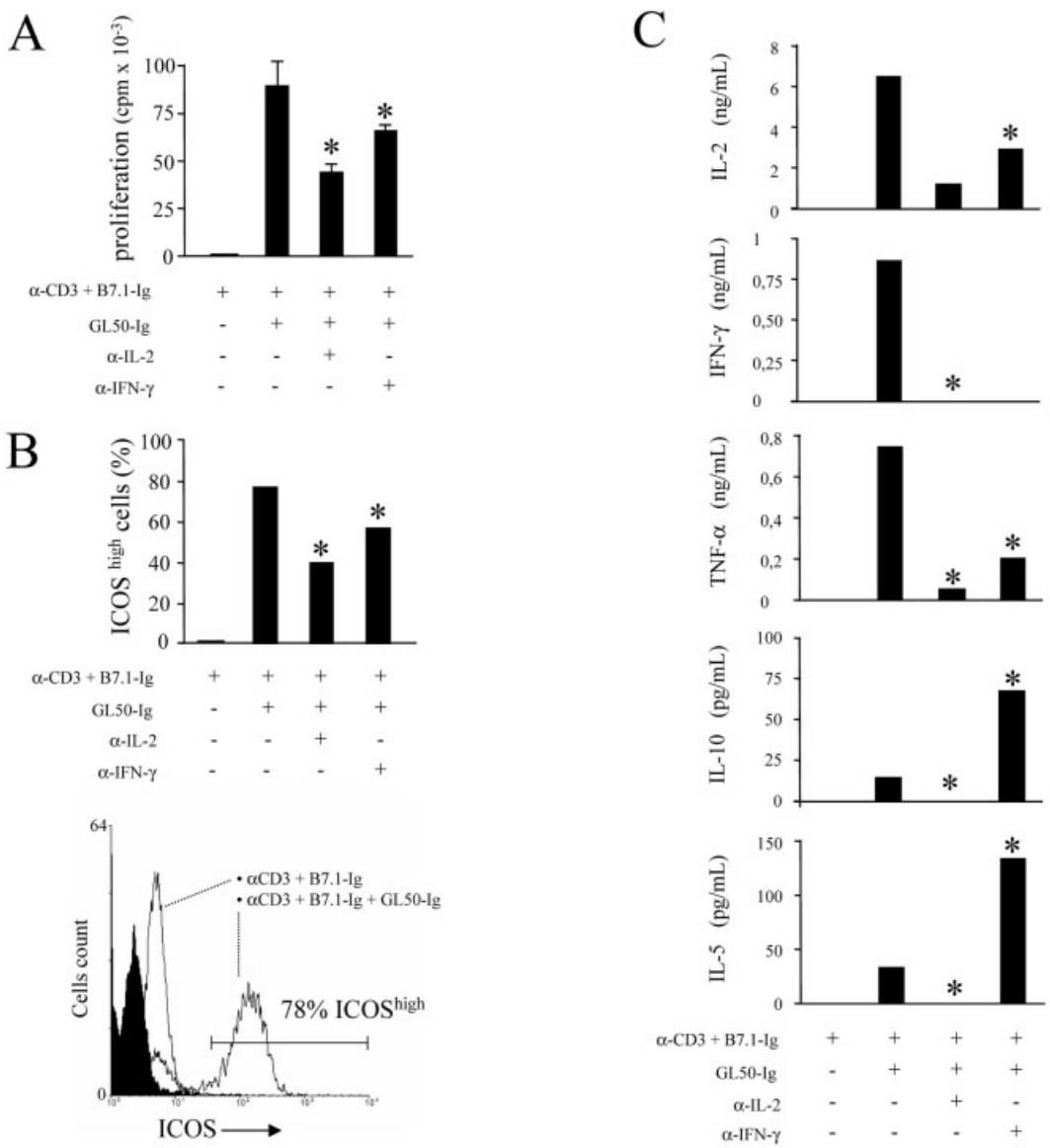

Figure 2. Role of IFN-γ and IL-2 in ICOS-mediated effector functions of naïve CD4$^+$ T cells. Naïve CD4$^+$ T were stimulated by immobilized anti-CD3 (CD3^{low}: 0.1 μg/mL) and B7.1-Ig (4 μg/mL) in the presence or absence of GL50-Ig (5 μg/mL), anti-IL-2 or anti-IFN-γ (20 μg/mL). (A) Cell proliferation was evaluated by [^{3}H]thymidine uptake during the last 6 h of 3-day cultures. (B) ICOS expression was evaluated on day 3 of culture, and is expressed as the percentage of cells expressing high levels of ICOS (ICOS^{high}), as shown by the cytofluorimetric histograms (open curves: ICOS expression in different activation conditions; shaded curve: negative control). (C) Cytokine assays were performed as in Fig. 1. Results are from one of four representative experiments. Error bars indicate the SD from triplicate cultures in the proliferation assays. Cytokine analysis was performed on mixed supernatants from triplicate cultures. * Significant differences *versus* the assay performed in the same conditions but in the absence of exogenous anti-IL-2 or anti-IFN-γ ($p<0.05$, Mann-Whitney test for paired samples calculated on the four experiments). Note that the statistical analysis does not include IFN-γ and IL-2 levels in the assays in which they were influenced by addition of the respective Ab.

Results showed that this treatment substantially inhibited ICOS expression (Fig. 3), whereas lower doses of IL-4 (0.2 ng/mL) had no effect (data not shown).

These results suggest that both IFN-γ and IL-2, but not IL-4, play a positive role in ICOS-mediated costimulation in the presence of suboptimal stimuli delivered through CD3+CD28.

Role of ICOS-induced costimulation in the absence of CD28 triggering

To further assess the role of IL-2 and IFN-γ in ICOS function, we used activation conditions independent from CD28, *i.e.*, activation of naïve Th cells driven by high doses of anti-CD3 mAb (CD3^{high}) plus ICOS triggering, which induce a weak activation of naïve

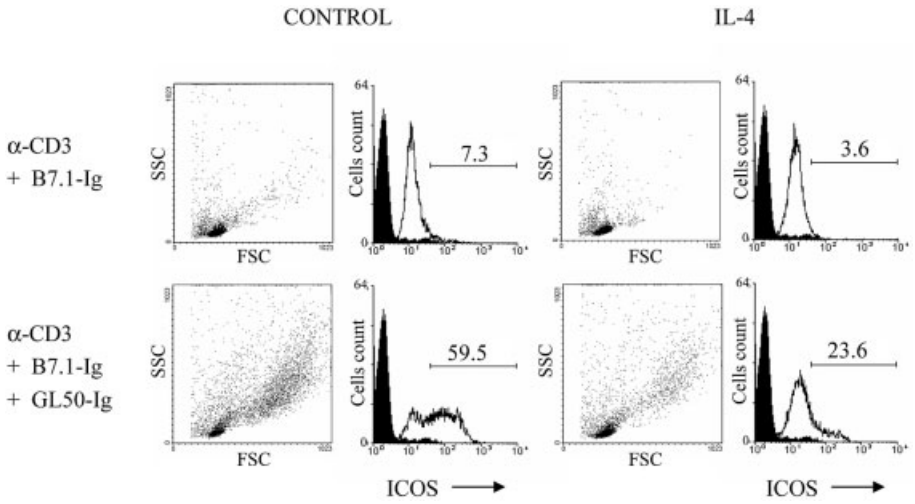

Figure 3. Effect of addition of IL-4 on expression of ICOS by naïve $CD4^+$ T cells. Naïve $CD4^+$ cells were stimulated by immobilized anti-CD3 ($CD3^{low}$: 0.1 μg/mL) and B7.1-Ig (4 μg/mL) in the presence or absence of GL50-Ig (5 μg/mL) and IL-4 (2 ng/mL). Expression of ICOS (open) or control (shaded) was assessed on day 3. The marker is set as in Fig. 2B, and the proportion of $ICOS^{high}$ cells is indicated by the numbers. Results are from one of three representative experiments.

$CD4^+$ T cells. Experiments were performed in the presence and absence of exogenous IFN-γ and IL-2 to find out whether their addition overcomes the incomplete cell activation induced by these stimuli. Moreover, anti-IFN-γ mAb was used to assess the role of its endogenous secretion.

In the absence of exogenous cytokines, ICOS triggering induced partial cell activation, characterized by cell proliferation (Fig. 4A) and up-regulation of ICOS (Fig. 4B) and CD25 (not shown), but secretion of only low amounts of IL-2, IFN-γ, IL-10 and TNF-α (Fig. 4C), which suggests acquirement of weak effector functions.

Addition of IL-2 strikingly increased proliferation, secretion of IL-10, IFN-γ, TNF-α and IL-2 itself, and expression of ICOS, with a pattern similar to that induced by the $CD3^{int}$+CD28+ICOS stimulation (Fig. 4).

Addition of IFN-γ moderately increased proliferation, secretion of TNF-α and IL-2, and ICOS expression, whereas IL-10 secretion was decreased (Fig. 4).

In the absence of exogenous IL-2, blocking of endogenous IFN-γ decreased cell proliferation and expression of ICOS, and the secretion of IL-2, TNF-α, and IL-10. By contrast in the presence of exogenous IL-2, it inhibited IL-2 and TNF-α secretion and increased IL-10 secretion, but had no effect on cell proliferation and ICOS expression.

Effect of ICOS-induced costimulation on differentiation of Treg

The striking IL-10 secretion induced by the $CD3^{high}$+ ICOS stimulus in the presence of exogenous IL-2 suggests that ICOS may favor differentiation of type 1 regulatory T cells (Tr1), which produce high levels of IL-10. To further assess the role of ICOS in Treg differentiation, we evaluated the effect of ICOS triggering on secretion of TGF-β1 and expression of Forkhead Box P3 (FoxP3), which characterize Th3 and naturally occurring $CD4^+CD25^+$ Treg, respectively.

TGF-β1 secretion was evaluated on cells activated by the $CD3^{high}$ or $CD3^{high}$+ICOS stimuli in the presence and absence of exogenous IL-2 and/or anti-IFN-γ mAb. The experiments were performed in serum-free medium since bovine serum contains TGF-β1. Results showed that stimulation *via* $CD3^{high}$ did not induce secretion of TGF-β1, but ICOS costimulation induced secretion of substantial amounts of this cytokine, which were further increased by addition of IL-2. Addition of anti-IFN-γ mAb increased TGF-β1 secretion by cells activated *via* $CD3^{high}$+ICOS (Fig. 5A).

FoxP3 expression was evaluated on cells stimulated *via* $CD3^{high}$ alone, $CD3^{high}$+ICOS or $CD3^{high}$+CD28 to compare the effect of the two costimulators. Results showed that the CD3+ICOS stimulation induced expression of high levels of FoxP3 comparable to those induced by CD28. They were not enhanced by exogenous IL-2, nor by contemporary triggering of CD3+ICOS+CD28. By contrast, stimulation of CD3 alone did not induce FoxP3 expression (Fig. 5B).

To directly evaluate whether ICOS stimulation favors differentiation of Treg, we assessed the suppressive activity of naïve $CD4^+$ T cells activated for 3 days with different stimuli on proliferation of fresh allogeneic naïve $CD4^+$ T cells driven by irradiated PBMC, that were autologous to the regulatory cells (Fig. 6). Results showed that naïve $CD4^+$ T cells stimulated *via* $CD3^{int}$ alone inhibited proliferation, as reported by other

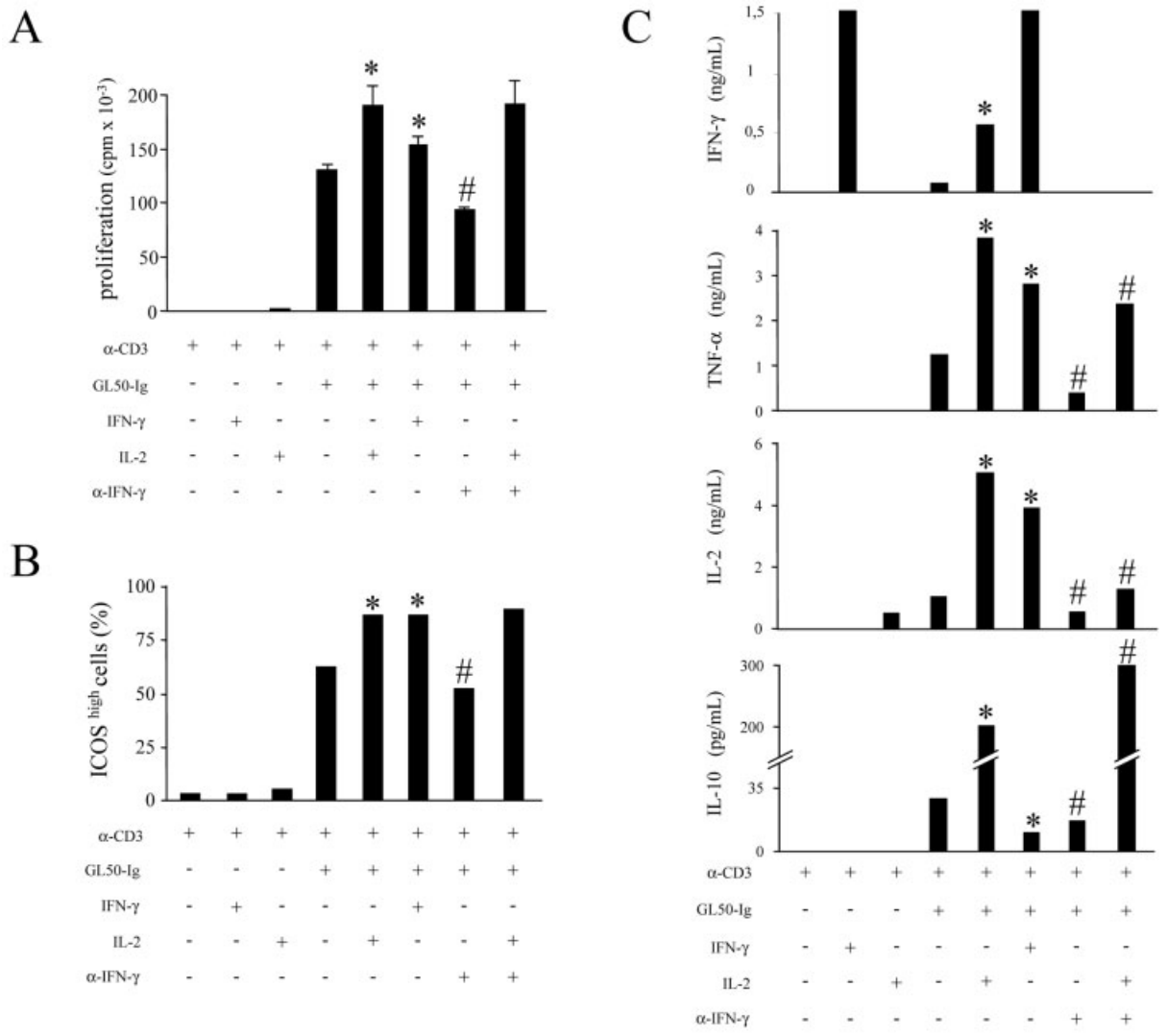

Figure 4. ICOS-mediated costimulation in the absence of CD28 stimulation. Naïve $CD4^+$ T cells were stimulated by immobilized anti-CD3 ($CD3^{high}$: 10 μg/mL) in the presence or absence of GL50-Ig (5 μg/mL), anti-IFN-γ (20 μg/mL), IFN-γ (80 ng/mL), and IL-2 (2 U/mL). (A) Cell proliferation, (B) ICOS expression, and (C) cytokine assays were performed as in Fig. 2. Results are from one of four representative experiments. Error bars indicate the SD from triplicate cultures in the proliferation assays. Cytokine analysis was performed on mixed supernatants from triplicate cultures. * Significant differences *versus* the assay performed in the same conditions but in the absence of IL-2 or IFN-γ; # significant differences *versus* the assay performed in the same conditions but in the absence of the anti-IFN-γ Ab ($p<0.05$, Mann-Whitney test for paired samples calculated on the four experiments). Note that the statistical analysis does not include IFN-γ levels in the assays were they were influenced by addition of exogenous IFN-γ and anti-IFN-γ Ab. By contrast, it includes all IL-2 evaluations since exogenous IL-2 minimally influenced the IL-2 levels.

authors [35], and inhibition was significantly increased by stimulation with $CD3^{int}$+ICOS. By contrast, unstimulated naïve $CD4^+$ T cells and those stimulated *via* $CD3^{int}$+CD28 did not exert any inhibitory effect. Addition of IL-2 to the $CD3^{int}$+ICOS stimulus decreased but not abolished the inhibitory activity.

Immature DC (iDC) express high levels of B7h, but low levels of B7.1 and B7.2 and have been reported to induce Treg [36]. To assess whether ICOS is involved in differentiation of these Treg, naïve $CD4^+$ T cells were incubated for 6 days with autologous iDC in the presence and absence of a soluble form of ICOS (ICOS-Ig) (1 μg/mL) to block the ICOS/B7h interaction. Then, we assessed the capacity of these cells to suppress the proliferation of fresh allogeneic naïve $CD4^+$ T cells driven by LPS-activated DC cells that were autologous to the regulatory cells. Results showed that incubation with iDC induced a substantial suppressive activity, that was impaired by ICOS-Ig (Fig. 7).

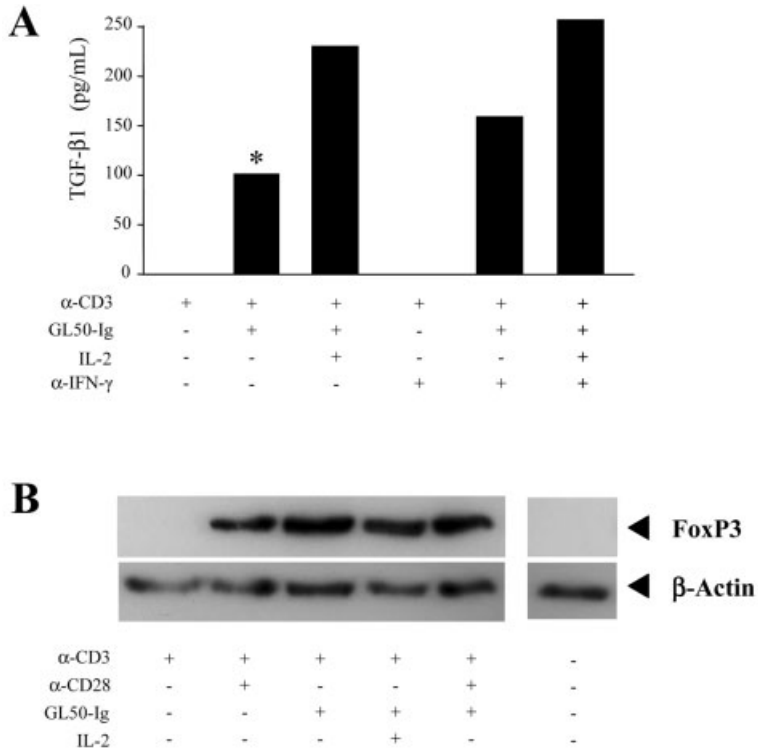

Figure 5. Effect of ICOS-mediated costimulation on secretion of TGF-β1 and expression of FoxP3. (A) Naïve $CD4^+$ T cells were stimulated by immobilized anti-CD3 ($CD3^{high}$: 10 μg/mL) in the presence or absence of GL50-Ig (5 μg/mL), anti-IFN-γ (20 μg/mL), and IL-2 (2 U/mL) and TGF-β1 secretion was evaluated after 3 days of culture. Cytokine analysis was performed on mixed supernatants from triplicate cultures. Results are from one of four representative experiments. (B) Naïve $CD4^+$ T cells were stimulated by immobilized anti-CD3 (10 μg/mL) in the presence or absence of GL50-Ig (5 μg/mL), anti-CD28 (4 μg/mL), and IL-2 (2 U/mL), and FoxP3 expression was evaluated after 3 days of culture by Western blotting. TGF-β1 secretion detected on cells stimulated with anti-CD3+GL50 (marked by *) was significantly different from each other activation condition ($p<0.05$, Mann-Whitney test for paired samples calculated on the four experiments).

Discussion

ICOS: A role in costimulation of human naïve T cells?

The aim of this work was to evaluate the role of ICOS in activation and differentiation of naïve Th cells and its cooperation with CD28-induced costimulation using the physiological ligands of these costimulatory molecules *in vitro*. The issue is crucial since, in most studies, (i) ICOS function has been evaluated in mouse cells or human total $CD4^+$ T cells, and (ii) ICOS has been stimulated by mAb, which may trigger signals partly different from those triggered by the natural ligands. In our hands, indeed, two anti-ICOS mAb recognizing partly distinct epitopes (*i.e.*, C398.4A and F44) displayed partly different effects on T cell activation (Mesturini, personal observation).

ICOS was previously envisaged as a costimulatory molecule predominantly involved in activation of memory T cells during the secondary immune response, whereas in the primary immune response activation of naïve T cells may be supposed to predominantly involve CD28. In line with this possibility, ICOS is almost absent in naïve T cells and several groups have shown that ICOS-induced costimulation does not induce secretion of IL-2, and hence cannot support Th precursor cell expansion.

ICOS costimulates differentiation of Th effector cells by acting in cooperation or not with CD28

Our results show that ICOS may act as a third signal allowing full activation and differentiation of human naïve Th cells stimulated *via* CD3/TCR and CD28. Moreover, it may be the key switching-factor triggering cell activation when it cooperates with suboptimal stimuli unable to induce full cell activation, such as suboptimal stimulation of CD3+CD28 ($CD3^{low}$+CD28) or stimulation of CD3 alone in the presence of exogenous IL-2 ($CD3^{high}$+IL-2).

In cells activated with optimal stimulation of CD3 and CD28 ($CD3^{int}$+CD28), triggering of ICOS displayed a minor effect on proliferation, and CD25 and ICOS expression, which were already strongly induced, but it potently increased secretion of the pro-inflammatory cytokines IFN-γ, TNF-α and IL-2, the anti-inflammatory cytokine IL-10, and the Th2 cytokine IL-5. This suggests that ICOS triggering is crucial to allow full activation of Th effector cells. This possibility is supported by data on ICOS- and B7h-deficient mice showing defective T cell proliferation and cytokine secretion. It is also in line with the findings of Wassink *et al.* [33] that ICOS potentiates cytokine secretion in both Th1- and Th2-oriented cell lines.

In the absence of CD28 triggering, stimulation of ICOS+CD3 activates cells only partially by inducing expression of ICOS and CD25, but with minimal proliferation and cytokine secretion. This partial

unresponsiveness can be overcome by addition of IL-2, which seems to be crucial for ICOS function. This possibility is confirmed by the observation that ICOS-induced costimulation of cells activated *via* CD3+CD28 is almost completely abolished by neutralization of endogenous IL-2 in the culture medium. These data suggest that the CD28-mediated sensitization of ICOS function is mainly mediated by IL-2, and that high

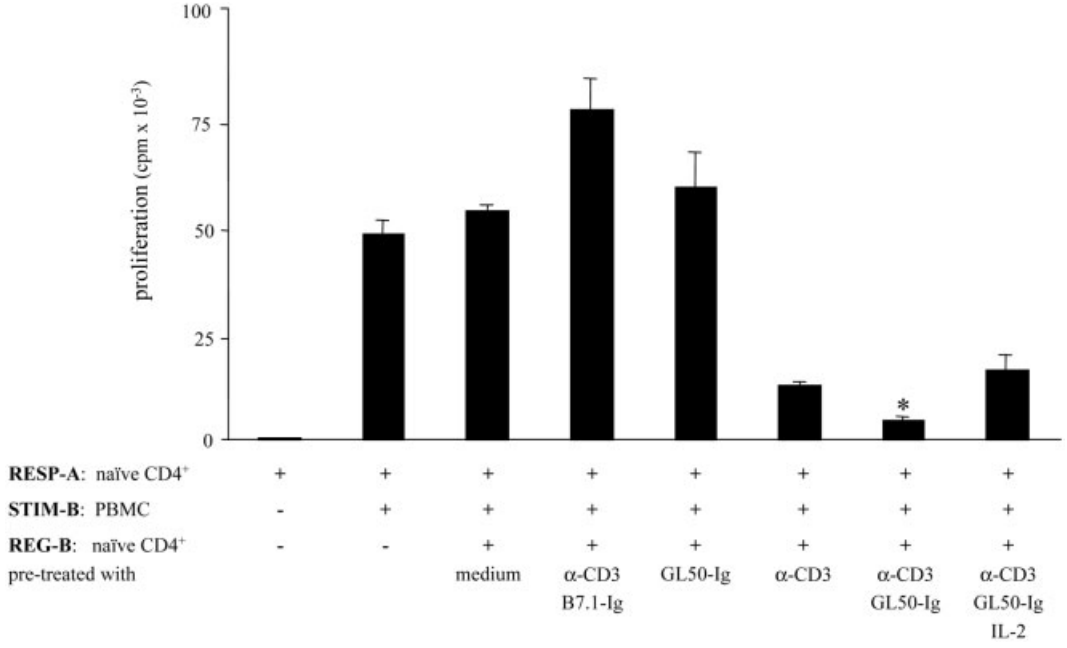

Figure 6. Regulatory activity of naïve CD4+ T cells preactivated with different stimuli on proliferation of naïve CD4+ T cells induced by allogeneic PBMC. Naïve CD4+ T cells from donor-A (5×10^4, responders: RESP-A) were cultured for 6 days in the absence and presence of 10^5 irradiated allogeneic PBMC from donor-B (stimulators: STIM-B) and 5×10^4 autologous naïve CD4+ cells from donor-B (regulators: REG-B) pre-cultured for 3 days in the presence of medium alone or anti-CD3 mAb ($CD3^{int}$: 1 μg/mL) or anti-$CD3^{int}$+B7.1-Ig (4 μg/mL), GL50-Ig (5 μg/mL), anti-$CD3^{int}$+GL50-Ig, anti-$CD3^{int}$+GL50-Ig+IL-2 (2 U/mL). Proliferation was measured at day 6. Results are from one of four representative experiments. * Significant difference *versus* the suppression detected using cells pre-treated with anti-CD3 alone ($p<0.05$, Mann-Whitney test for paired samples calculated on the four experiments).

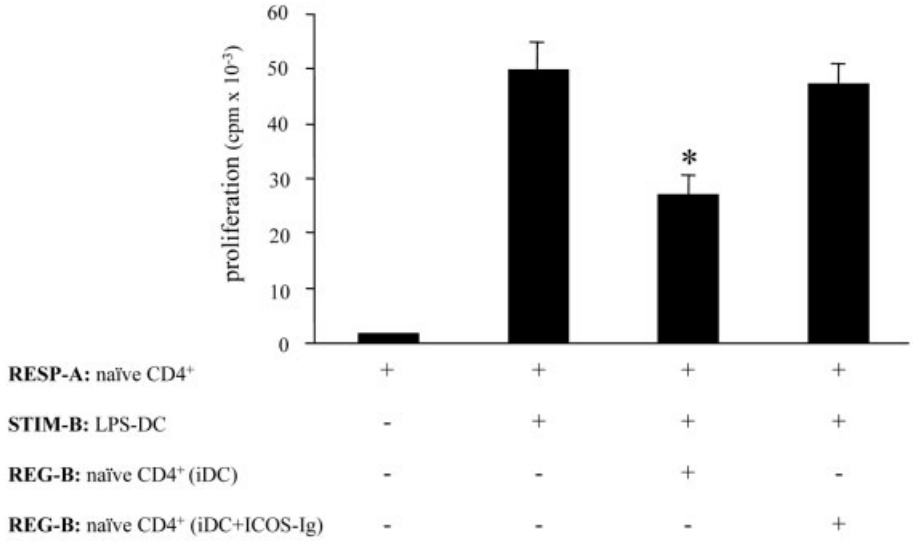

Figure 7. Regulatory activity of naïve CD4+ T cells precultured with iDC on proliferation of naïve CD4+ T cells induced by allogeneic LPS-activated DC. Naïve CD4+ T cells from donor-A (5×10^4, responders: RESP-A) were cultured for 6 days in the absence and presence of 5×10^4 allogeneic LPS-DC from donor-B (stimulators: STIM-B) and 5×10^4 autologous naïve CD4+ cells from donor-B (regulators: REG-B) pre-cultured for 6 days with iDC from donor-B in the presence and absence of ICOS-Ig (1 μg/mL). Proliferation was measured at day 6. Results are from one of four representative experiments. * Significant difference *versus* the assay performed in the absence of REG-B ($p<0.05$, Mann-Whitney test for paired samples calculated on the four experiments).

microenvironmental levels of IL-2 may overcome the need for CD28 triggering and favor ICOS-induced recruitment of antigen-specific naïve T cells during the primary immune response.

ICOS builds a positive feedback loop with IFN-γ and IL-2 secretion

IL-4 secretion was never detected under any culture conditions and addition of IL-4 inhibited ICOS expression, which is in line with data obtained in human cells by Wassink *et al*. This finding is in contrast with data obtained in the mouse showing that ICOS and IL-4 build up a positive feedback loop in which they reciprocally stimulate each other's expression [31, 32]. By contrast in our human model, a similar loop seems to involve ICOS and IFN-γ; this effect needs the presence of IL-2 to sensitize cells to ICOS-induced costimulation. This model is in line with the demonstration by Wassink *et al*. that ICOS expression is potentiated by IL-12, a potent inducer of IFN-γ secretion, which suggests that ICOS-induced costimulation favors differentiation towards Th1 more than Th2 cells in human naïve Th cells.

The strong effect of ICOS-induced costimulation was surprising in light of the weak expression of ICOS in unstimulated naïve $CD4^+$ T cells, but can be explained by the fact that up-regulation of ICOS expression is detected as early as 12–24 h upon primary stimulation. This up-regulation is not detectable in cells activated by stimulation of CD3 alone, even in the presence of exogenous IL-2, and is strictly dependent on costimulation of either CD28 or ICOS. IL-2 is, nevertheless, necessary since its neutralization inhibits ICOS up-regulation.

ICOS favors differentiation of Treg

A different point is the effect of ICOS on induction of naïve $CD4^+$ T cells toward Treg, which is relevant to the question whether Treg are generated from naïve T cells or Th1 and Th2 cells that have undergone chronic stimulation [21, 37–42]. This possibility is suggested by the finding that ICOS costimulates secretion of IL-10, particularly in the presence of high levels of IL-2 and in the absence of IFN-γ. It is noteworthy that secretion of both IL-10 and IFN-γ is typical of Tr1 [43]. Moreover, ICOS induced expression of TGF-β1 and FoxP3, typical of naturally occurring $CD4^+CD25^+$ Treg, together with expression of IL-10 [21, 37–42]. Intriguingly, expression of TGF-β1 and FoxP3 was induced at substantial levels, even by stimulation of CD3+ICOS in the absence of the support given by CD28 triggering or exogenous IL-2, when minimal levels of most cytokines were induced. Finally, direct evaluation of the regulatory activity of these cells showed that triggering of CD3+ICOS induced a striking regulatory activity that was higher than that induced by triggering of CD3 alone, previously described by other authors [35]. This regulatory activity was not induced by CD3+CD28 stimulation and was only partly inhibited by addition of exogenous IL-2. These findings suggest that, in the presence of insufficient stimuli for activation, ICOS triggering favors differentiation of cells with regulatory functions. This possibility is in line with the notion that iDC, expressing B7h, but low levels of the CD28 ligands, induce a potent T cell regulatory activity [36]. Our experiments confirmed those data and showed that blocking of the ICOS/B7h interaction in this system inhibits induction of Treg. In line with this model, ICOS triggering seems to play a role in the unresponsive state induced by exposure of mice to inhaled ovalbumin in the absence of costimulatory signals induced by adjuvants; this unresponsiveness seems to involve Treg and IL-10 [21, 40].

Conclusions

In conclusion, this work shows that ICOS may deliver a third signal for T cell activation and this may have two roles. On one hand, it may allow full differentiation of effector cells in the presence of optimal stimulation of the CD3/TCR (first signal) and CD28 (second signal), on the other hand it may be the switching factor for activation when the CD28-induced costimulation is weak or absent by acting in synergy with the CD28 signal and/or with the IL-2 present in the microenvironment.

In this connection, it is noteworthy that B7h is constitutively expressed by several cell types of both hematopoietic and non-hematopoietic origin, whereas B7.1 and B7.2 are expressed by activated APC only. Therefore, ICOS may play a crucial role when (i) B7.1 and B7.2 are weakly expressed because professional APC are not fully activated, or (ii) IL-2 is abundant in the microenvironment, because of its secretion by bystander activated lymphocytes, in the presence of non-professional APC expressing B7h but not B7.1 and B7.2. The positive feedback loop between IFN-γ and ICOS suggests that NK cells may support ICOS function in the early phase of the immune response, when activated NK are the main source of IFN-γ [44]. Therefore, ICOS may allow responses to weak antigens in the context of immune response against strong antigens. This may be significant in the development of autoimmune diseases in which the response to weak autoantigens can be favored by responses against strong infectious antigens. An opposite aspect is that ICOS can oppose inappropriate activation of the immune response, when activation stimuli are insufficient because of the lack of CD28-induced costimulation by inducing differentiation of Treg.

Materials and methods

Cell purification and immunofluorescence analysis

PBMC were isolated by gradient centrifugation of buffy coats from the local blood bank. Naïve $CD4^+$ T cells were purified by panning to remove $CD11b^+$, $CD45RO^+$ and $HLA\text{-}DR^+$ cells with the appropriate mAb, followed by use of the $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany). This approach provided >97% cells displaying the phenotype $CD3^+CD4^+CD45RA^+CD14^-CD16^-$, as assessed by flow cytometry.

The following mAb were used in the panning technique: OKM1 (CD11b), L243 (HLA-DR), UCHL1 (CD45RO). They were purified by affinity chromatography on protein G-Sepharose 4 fast-flow columns (Amersham Biosciences GmbH, Freiburg, Germany) from the hybridoma supernatants.

The following mAb were used in the immunofluorescence experiments: PE-conjugated anti-CD3 (Sigma-Aldrich, St. Louis, MO), -CD25, -CD8, -CD14, HLA-DR (Caltag, Burlingame, CA), -CD56 (Becton Dickinson, San Jose, CA), and FITC-conjugated anti-CD45RA, -CD45RO, -CD4 (Sigma), -CD19 (Caltag), -CTLA-4 (R&D System, Minneapolis, MN), ICOS was stained with C398.4A (purified as described above) plus FITC-conjugated goat anti-hamster Ig pre-absorbed with mouse and rat Ig (Caltag). Production of the C398.4A mAb (hamster IgG) has been previously reported [45]. Samples were analyzed with a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson). Antigen expression was evaluated as percentage of positive cells.

Cell activation and cytokine analysis

The cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS. Flat-bottom 96-well microplates were first coated with 100 μL of the indicated concentrations of anti-CD3 mAb (OKT3) overnight at 4°C. After washing with PBS, the plates were further coated with the indicated concentrations of recombinant human B7-1/Fc chimera (R&D Systems), human GL50-mIg (R&D System) and anti-CTLA-4 (Ancell, Bayport, MN) mAb at room temperature for 2 h. Plates were then washed with PBS, purified naïve $CD4^+$ T cells were seeded to the wells at 1×10^5 cells/well in triplicate in 200 μL RPMI 1640+10% FBS. Cells were cultured for 72 h and [^{3}H]thymidine (1 μCi/well; Amersham) was added during the last 6 h; cells were then harvested and [^{3}H]thymidine uptake was evaluated with a β-counter (Wallac-Perkin Elmer, Boston, MA).

To analyze cytokines, supernatants were collected after 65 h of culture and concentrations of IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ were evaluated with the human Th1/Th2 CBA kit (BD Biosciences). Concentration of TGF-β1 was evaluated by ELISA (R&D Systems) according to the manufacturer's instructions; in these experiments cells were cultured in the serum-free medium AIM-V (Gibco, Invitrogen, Gaithersburg, MD).

Anti-IFN-γ (20 μg/mL), anti-IL-2 (20 μg/mL) Ab, recombinant human IFN-γ (80 ng/mL), and IL-4 (2 ng/mL) were purchased from R&D Systems, and IL-2 (2 U/mL) from Sigma-Aldrich.

Function of Treg was assessed using a standard suppression assay of proliferation. Briefly, naïve $CD4^+$ T cells (from donor A) were used as responder cells (5×10^4 cells/well) in 6-day cultures in 96-well round-bottom microplates in the presence of 10^5 irradiated (2000 rad) allogeneic PBMC (from donor B) used as stimulator cells and naïve $CD4^+$ cells (5×10^4 cells/well from donor B) pre-activated for 3 days with different stimuli, used as regulatory cells. Proliferation was measured as reported above at day 6.

In some experiments, regulatory cells (from donor B) were induced by culturing naïve $CD4^+$ T cells for 6 days with autologous (from donor B) iDC using a stimulator/responder ratio of 1:20 in the presence and absence of ICOS-Ig (1 μg/mL) (R&D System, Minneapolis, MN). These cells (5×10^4 cells/well) were used to suppress the proliferation of allogeneic (from donor A) naïve $CD4^+$ T cells (5×10^4 cells/well) induced by LPS-activated DC (LPS-DC) (5×10^4 cells/well) that were autologous to the regulatory cells.

To obtain iDC, PBMC were allowed to adhere to plastic dishes for 2 h. Adherent cells were cultured for 5 days in RPMI 1640+10% FBS in the presence of recombinant human GM-CSF (800 U/mL; PeproTech, London, UK) and rhIL-4 (10 ng/mL). To obtain LPS-DC, iDC were further cultured for 2 days in the presence of LPS serotype 055:B5 (1 μg/mL; Sigma). Immunofluorescence analysis showed that iDC were $CD83^-$ and LPS-DC were $CD83^+$.

Western blot analysis

Cells (2×10^6/well) were cultured in 2 mL medium in a six-well plate precoated with the appropriate stimuli as described. The anti-CD28 mAb (clone ANC28.1/5D10) was obtained from Ancell (Bayport, MN). After culture, cells were washed twice with PBS, lysed for 20 min on ice in AKT buffer (10 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5, 1% Triton X-100, 1% sodium deoxycholate, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 100 μg/mL PMSF). Lysates were then cleared by centrifugation for 10 min at 13 000 rpm at 4°C, and separated on 10% SDS-PAGE gels after denaturation in SDS-PAGE loading buffer (63 mM Tris-HCl pH 6.8, 5% glycerol, 1% SDS, 2.5% bromophenol blue). Gel was transferred to nitrocellulose and filters were blocked in TBST buffer plus 5% non-fat milk for 1 h, and then incubated with 2 μg/mL goat anti-human FoxP3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C in TBST buffer plus 5% BSA. FoxP3 signals were then revealed with an anti-goat HRP-labeled secondary antibody (1:400, Santa Cruz Biotechnology) and the ECL system (Amersham). β-Actin was subsequently detected as an internal control with mouse anti-β-actin antibody (Sigma-Aldrich) and HRP-labeled secondary antibody (1:3000, Amersham Biosciences).

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Risultati (2)

L'INTERAZIONE DI B7h CON ICOS INDUCE IL-1β/IL-23 NELLE CELLULE DENDRITICHE E IL-17/IL-22 NEI LINFOCITI T_H

Per meglio comprendere il ruolo complessivo del sistema recettoriale ICOS/B7h ho voluto valutare gli effetti del *reverse signaling* di B7h sui diversi tipi di APC, ovvero cellule dendritiche, macrofagi e linfociti B. Poiché in esperimenti preliminari i risultati più interessanti sono stati ottenuti sulle cellule dendritiche (DC), mi sono poi concentrata su questo tipo cellulare.

La stimolazione di B7h sulle DC è stata simulata per mezzo di un proteina di fusione (ICOS-Ig) costituita dalla porzione extracellulare di ICOS coniugata al frammento Fc di una IgG umana. Questi esperimenti hanno dimostrato che ICOS-Ig modula in modo sostanziale la produzione citochinica da parte di DC attivate con LPS, riducendo la produzione di TNFα e potenziando la produzione di IL-10 e soprattutto di IL-1β. A livello della produzione di IL-1β, abbiamo osservato che LPS induce la produzione di un precursore inattivo citoplasmatico della citochina, mentre ICOS-Ig induce l'attivazione di caspasi-1, la quale è poi responsabile della scissione proteolitica del precursore e della conseguente secrezione della forma attiva della citochina. Una analisi della capacità di queste DC di attivare i linfociti T in vitro in colture miste linfocitarie ha dimostrato che la stimolazione di ICOS-Ig modula in modo sostanziale l'attività stimolatoria delle DC, inducendo l'attivazione di linfociti che producono aumentate quantità di IL-17, una citochina prodotta in modo caratteristico dai linfociti T_H17. Mediante esperimenti di antagonismo abbiamo dimostrato che la differenziazione dei T_H17 in questo sistema dipende sia dalla produzione di IL1-β da parte delle DC sia dalla stimolazione di ICOS sui linfociti T. Pertanto questi esperimenti indicano che l'interazione ICOS/B7h può essere coinvolta in modo bidirezionale nell'induzione della differenziazione dei T_H17: da un lato la stimolazione di B7h induce la produzione di IL-1β da parte delle DC, necessaria per questa differenzazione, dall'altro la stimolazione di ICOS sui linfociti T sostiene questa differenziazione.

INTERACTION OF B7h WITH ICOS TRIGGERS IL-1β/IL-23 IN DENDRITIC CELLS AND IL-17/IL-22 IN Th CELLS

Annalisa Chiocchetti[1,6], Riccardo Mesturini[1,6], Stefania Nicola[1], Elena Boggio[1], Ilaria Seren Bernardone[1], Chiara Dianzani[2], Alessandro Poggi[3], Jungi Yagi[4], Josè Maria Rojo[5], Umberto Dianzani[1]

[1]Interdisciplinary Research Center of Autoimmune Diseases (IRCAD) and Department of Medical Sciences, "A. Avogadro" University of Eastern Piedmont, Novara, Italy.

[2]Department of Anatomy, Pharmacology and Forensic Medicine, University of Turin, Torino, Italy.

[3]Laboratory of Immunology, Department of Translational Oncology, National Institute for Cancer Research, Genoa, Italy

[4]Department of Microbiology and Immunology, Tokyo Women's Medical University, Tokyo, Japan.

[5]Department of Cellular and Molecular Physiopathology, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain

[6]These authors contributed equally to this work

ABSTRACT

B7h, expressed by several cell types, is the ligand of the Inducible Costimulator (ICOS), expressed by activated T cells. We show that B7h triggering induces interleukin IL-1β/IL-23 secretion in LPS-activated dendritic cells (DC), that in turn induces IL-17/IL-22 secretion in allogeneic mixed lymphocyte cultures (MLC). This B7h triggering was the "second signal" for IL-1β secretion by activating caspase-1 and cleaving the IL-1β precursor. ICOS triggering also plays a role in IL-17/IL-22 secretion, since this is inhibited in these MLC by blockade of the ICOS:B7h interaction, and induced in CD3-stimulated naïve T helper cells by ICOS triggering in the presence of IL-1β. Therefore, the ICOS:B7h interaction between DC and T helper cells triggers bidirectional signals that cooperate to induce Th17 polarization.

Full activation of naïve T cells requires their receipt of three signals from antigen-presenting cells (APC)[1]. The first is delivered through the TCR upon recognition of the antigenic peptide presented by the appropriate MHC molecule, the second is provided by T cell costimulatory molecules engaged by their ligands expressed on APC, and the third is delivered by cytokines in the microenvironment[2]. In the absence of these signals, T cells

become anergic or die by apoptosis[3]. CD28, the best-known T cell costimulatory molecule, is constitutively expressed by T cells, binds B7.1 (CD80) and B7.2 (CD86) onAPC, and promotes T cell proliferation and cytokine secretion[4]. CTLA-4 belongs to the CD28 family and binds the same ligands as CD28, but transduces negative signals down-modulating T cell activation. It has recently been shown that B7.1 and B7.2, too, transduce signals upon engagement by CD28 or CTLA-4 and modulate APC function: this has been described as "reverse signalling"[5].

Reverse signalling triggered by a CD28-Ig fusion molecule leads to production of IL-6 from mouse DC and thus results in their activation, whereas CTLA-4-Ig up-regulates indoleamine 2,3-dioxygenase, and degrades tryptophan to products that inhibit T cell proliferation[6]. Other members of the CD28 family are ICOS, PD-1, and BTLA7-10, whereas other members of the B7 family are B7h, B7H1, B7H3, B7H4, and B7-DC8, [11-14.]

ICOS (CD278) is a costimulatory molecule selectively expressed by activated T cells. It binds B7h (CD275, also known as B7H2, B7-RP1, ICOSL, GL50) expressed by both haematopoietic and nonhaematopoietic cells. B7h is constitutively expressed by B cells, macrophages, DC, and a subset of mouse spleen T cells. In monocytes, expression is up-regulated upon activation. B7h is also expressed by vascular endothelial cells, epithelial cells, and fibroblasts[3,15-21]. This expression pattern suggests that the ICOS:B7h interaction has a role in both T cell activation in lymphoid organs, and the control of T cell function at sites of inflammation. ICOS function has been extensively investigated in T cells, where it seems to modulate cytokine secretion by predominantly acting on recently activated/memory cells, whereas it is less effective in naïve T cells, whose activation and IL-2 secretion seems to mostly depend on CD28 costimulation[3,7,8,22-24]. Moreover, substantial differences in ICOS function have been reported between mouse and human cells. In mice, ICOS triggering has been predominantly related to induction of IL-4-secreting Th type 2 (Th2) cells, or IL-10-secreting regulatory T (Treg) cells, but it can also induce IFN-γ-secreting Th type 1 (Th1) cells in some circumstances[25]. In humans, we have recently reported that ICOS-mediated costimulation of naïve Th cells elicited different responses depending on the cytokine milieu, since it promoted IFN-γ secretion in the presence, and IL-10 and TGF-β secretion in the absence of IL-22[6].

Primary aim of this work was to assess an unexplored different aspect of ICOS function, i.e. the reverse signaling, triggered through B7h, on DC.

In peripheral tissues, immature DC (iDC) are specialized in antigen uptake and processing for MHC presentation[27,28]. In the presence of inflammatory mediators, such as Toll-like

receptor ligands and inflammatory cytokines, iDC differentiate to mature DC capable of activating T cells, and characterized by decreased endocytic activity, increased expression of costimulatory and class II MHC molecules, and increased cytokine secretion[29-30]. Mature DC are at least 100 times more potent than macrophages in activating naïve T cells in vitro. In this study, we show that triggering of B7h substantially modulates Lypopolysaccharide (LPS) induced DC maturation by influencing cytokine secretion. The most striking effect was an 80-fold increase of IL-1β secretion, where B7h triggering was the "second signal" that activated caspase-1[31]. This substantially modulated the ability of DC to activate allogeneic T cells in mixed lymphocyte cultures (MLC), and strikingly increased secretion of IL-17. This effect was partly ascribable to IL-1β secretion, since it was reproduced by adding exogenous IL-1β to LPS-activated DC. Moreover, we found that ICOS triggering, too, played a role in IL-17 secretion, since blockade of the B7h:ICOS interaction inhibited IL-17 secretion in the MLC system. Indeed, in naïve Th cells activated with anti-CD3 monoclonal antibodies (mAb), ICOS costimulation induced Th17 differentiation in the presence of IL-1β or IL-1β+IL-6.

RESULTS

Effect of B7h stimulation on DC maturation

Peripheral blood mononuclear cells (PBMC) were induced to differentiate to iDC by a 5-day culture with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. Figure 1a shows their cytofluorimetric characterization. Monocytes expressed CD14 and B7.2, but not CD1a, B7.1, and B7h; in iDC, CD1a, B7.1, and B7h were up-modulated, CD14 was down-modulated, and B7.2 unchanged.

Since activation stimuli, such as LPS, induce iDC maturation to mDC, we assessed the effect of B7h stimulation in this process by using ICOS-Ig to trigger B7h. iDC were treated for 2 days with control medium (DCCTR), LPS (DCLPS), ICOS-Ig (DCICOS), or LPS plus ICOS-Ig (DCLPS/ICOS) and then we compared their surface marker expression, ability to capture antigens, allostimulatory activity, and cytokine secretion.

Analysis of surface expression of B7h, B7.1, B7.2, CD83, HLA-DR, and class I HLA, showed that, compared with DCCTR, DCICOS displayed mild up-regulation of B7.1 and HLA-DR and downmodulation of B7.2, whereas DCLPS up-regulated B7.1, B7.2, CD83, HLA-DR, and class I HLA. By contrast, DCLPS/ICOS displayed a pattern similar to DCLPS, but without substantial HLA-DR upregulation (Table 1).

Analysis of antigen capture was assessed evaluating uptake of FITC-conjugated dextran and showed that DCCTR and DCICOS displayed the same ability to capture antigens, and this activity was similarly down-regulated in DCLPS and DCLPS/ICOS (Fig. 1b).
Evaluation of the DC allostimulatory activity, by assessing the lymphocyte proliferative response in allogeneic MLC, showed that lymphocyte proliferation induced by DCCTR was similar to that induced by DCICOS, but lower than that induced by DCLPS (Fig. 1c). Moreover, proliferation induced by DCLPS/ICOS was higher than that induced by DCCTR, but lower than that induced by DCLPS.
Assessment of cytokine secretion by measuring levels of IL-1β, IL-6, IL-10, IL-12p70, IL-23, and TNF-α in the supernatants of the 2-day DC cultures showed that secretion of all these cytokines was minimal in DCCTR and DCICOS, whereas DCLPS secreted substantial amounts of TNF-α and IL-6, and, to a lesser extent, IL-10 and IL-12p70. Intriguingly, DCLPS/ICOS displayed a different cytokine secretion pattern from DCLPS, with higher secretion of IL-1β (80 fold), IL-23 (25 fold) and IL-10 (3 fold), and lower secretion of TNF-α (2 fold), whereas secretion of IL-12p70 and IL-6 was similar (Fig. 2a).
Kinetic experiments assessing IL-1β and IL-23 secretion 4, 6, 18, 24, and 48 h after stimulation showed that their up-modulation was already detectable in DCLPS/ICOS after 6 h of culture, and reached a plateau after 24 h (Fig. 2b).

B7h triggering acts as a "second signal" for IL-1β secretion

Secretion of IL-1β requires two signals[31,32]. The first senses the pathogen and induces synthesis of the 31 KDa inactive precursor pro-IL-1β. The "costimulatory" second signal is required to induce aggregation of a multimolecular complex, called the "inflammasome", which activates caspase-1, also known as IL-1-converting enzyme (ICE), which in turn proteolytically processes pro-IL-1β to generate the 17 KDa secreted active form of IL-1β.
To dissect the effect of B7h triggering on this pathway, we analyzed pro-IL-1β expression in the cell lysates (by Western blot), and the presence of the active forms of caspase-1 (by Western blot) and IL-1β (by enzyme-linked immunosorbent assay - ELISA), which are both rapidly secreted, in the supernatants of DCCTR, DCICOS, DCLPS, and DCLPS/ICOS 30 h after delivery of the respective activation signals (Fig. 3a). Results showed that DCLPS displayed striking induction of pro-IL-1β expression, but no caspase-1 activation and minimal IL-1β secretion, whereas DCICOS displayed no induction of pro-IL-1β expression and secretion of active IL-1β, but substantial activation of caspase-1. Lastly,DCLPS/ICOS

displayed a fully activated pattern, with induction of pro-IL-1β expression, caspase-1 activation, and active IL-1β secretion.

To assess the relationship between caspase-1 activation and IL-1β secretion, we evaluated the effect, exerted on IL-1β secretion, by the addition of YVAD or KCl to the DCLPS/ICOS cultures; YVAD inhibits caspase-1, whereas KCl antagonizes the cell K+ efflux required for inflammasome activation[33]. Results showed that both YVAD and KCl inhibited secretion of IL-1β by DCLPS/ICOS (Figure 3b). To assess specificity of the ICOS effect, we compared IL-1β secretion in DC treated for 2 days with LPS plus either CTLA-4-Ig, or recombinant Fcγ1 fragments, or total human IgG. Results showed that IL-1β secretion was induced by ICOS-Ig, none of the other stimuli was effective (Fig. 3c).

Since IL-18 is secreted through the same pathway, we also evaluated its secretion in the supernatants of DCCTR, DCICOS, DCLPS, and DCLPS/ICOS by ELISA, but no secretion was detected (data not shown).

DCLPS/ICOS induces IL-17 secretion in allogeneic MLC

IL-1β, IL-6 and IL-23 play a key role in differentiation of human Th17 cells which secrete IL-17 and IL-22, and expressing retinoic acid-related orphan nuclear receptor c (RORc) and retinoic acid-related orphan nuclear receptor a (RORa)[34]. To assess whether differences in IL-1β secretion displayed by DCLPS and DCLPS/ICOS paralleled the difference in their ability to polarize Th cell response, we evaluated secretion of Th1 (IL-2, IFN-γ), Th2 (IL-4, IL-5), Th17 (IL-17, IL-22) and Tr1 (IL-10, TGF-β) cytokines in the supernatants from MLC induced by coculturing allogeneic peripheral blood lymphocytes (PBL) with either DCLPS or DCLPS/ICOS. Compared to DCLPS-driven MLC, DCLPS/ICOSdriven MLC displayed some changes in IL-2, IL-5, IL-4, and IL-10, which were secreted at relatively low levels, and substantial down-modulation of IFN-γ and up-modulation of IL-17 and IL-22 (Fig. 4a).

The different activity displayed by DCLPS/ICOS compared to DCLPS was not due to decreased interaction between ICOS on T cells and B7h on DC as a consequence of B7h down-modulation or blocking by the ICOS-Ig used to prepare DCLPS/ICOS. Staining with anti-B7h mAb showed, in fact, similar expression levels in DCLPS and DCLPS/ICOS (Table 1); moreover, staining of these cells with anti-human Ig antibodies did not disclose any residual ICOS-Ig bound on the cell surface (data not shown).

Experiments assessing the ability of DCCTR and DCICOS to elicit cytokine secretion in allogeneic MLC detected minimal amounts of these cytokines with no substantial differences between DCCTR and DCICOS (data not shown).

To assess whether the high levels of IL-1β secreted by DCLPS/ICOS were responsible for the increased IL-17 secretion in MLC, we evaluated the effect on this secretion of the addition of exogenous IL-1β to the DCLPS-driven MLC and an anti-IL-1β antibody to the DCLPS/ICOS-driven MLC. Results showed that addition of IL-1β induced IL-17 secretion in the former, and IL-1β blockade inhibited its secretion in the latter (Fig. 4b).

ICOS triggering favours differentiation of Th17 cells.

These experiments indicate that B7h triggering on LPS-activated DC increases their IL-1β secretion, and hence their ability to induce IL-17 secretion by lymphocytes. However, since ICOS is known to trigger signals modulating cytokine secretion in T cells, we also determined whether its costimulation cooperates in this induction of IL-17. For this purpose, we performed the MLC assay in the presence and absence of ICOS-Ig in order to block the interaction between B7h on DCLPS/ICOS and ICOS on T cells. Results showed that ICOS-Ig strikingly inhibited IL-17 (Fig. 4c). This suggested that costimulatory signals transduced by ICOS in T cells play a role in induction of these cytokines. To confirm this possibility, we evaluated the effect of ICOS triggering on naïve CD4+ T cells activation driven by anti-CD3 mAb. Purified naïve CD4+ T cells were incubated in wells pre-coated with anti-CD3 mAb in the presence and absence of B7h-Ig, to trigger ICOS, and IL-1β, or IL-1β+IL-6, known to promote human Th17 differentiation; these experiments were performed in the presence of anti-IFN-γ and anti-IL-4 antibodies to inhibit Th1 and Th2 cell differentiation. In parallel, the effect of costimulation with B7.1-Ig or anti-CD28 mAb was assessed as a control. Results showed that ICOSmediated costimulation was more effective than CD28-mediated costimulation in inducing IL-17 secretion in the presence of IL-1β, and that this effect was even more pronounced when IL-1β was used in combination with IL-6 (Fig. 5a). By contrast, ICOS and CD28 triggering were similarly ineffective in inducing IL-17 secretion in the absence of the exogenous cytokines. These differences were not due to a different ability to induce lymphocyte proliferation, which was similarly induced by CD28- and ICOS-mediated costimulation in the presence of the exogenous cytokines (data not shown).

Beside IL-17, Th17 cells also secrete IL-22 and express the RORc and RORa transcription factors[34].

Therefore, we performed kinetic experiments to compare the effect of ICOS costimulation on secretion of IL-17 and IL-22 by naïve CD4+ cells activated with the protocol described above; cytokine secretion was assessed at day 2, 3, 4, 5 of culture. Results showed that ICOS costimulation in the presence of IL-1β and, to a greater extent, IL-1β+IL-6 induced up-modulation of both cytokines which was detectable at day 3 and further increased in the following days (Fig. 5b). Moreover, we evaluated expression of the RORc and RORa mRNA in the lysates of these cells collected at day 5. Real-time PCR analysis showed that ICOS triggering up-modulated expression of both genes in the absence of the exogenous cytokines; up-modulation of RORc expression was strikingly increased by IL-1β+IL-6, but not IL-1β alone, whereas RORa expression was not further increased by exogenous cytokines (Fig. 5c).

DISCUSSION

This work shows that reverse signaling mediated by B7h modulates the DC response to LPS by influencing cytokine secretion and the ability to drive T cell differentiation. These changes substantially modulate the T cell response and favour Th17 polarization with IL-17 and IL-22 secretion by lymphocytes.

Analysis of surface marker expression, antigen uptake, and cytokine secretion showed that B7h triggering per se was unable to induce iDC differentiation to mature DC, whereas, as expected, this was induced by LPS, since it down-regulated antigen uptake and up-regulated expression of B7.1, B7.2, CD83, and MHC molecules, allostimulatory activity, and secretion of the pro-inflammatory cytokines TNF-α, IL-6, and IL-12. However, B7h triggering substantially modulated the LPS-induced cytokine secretion by increasing IL-1β and IL-23, and decreasing TNF-α secretion. These effects were not ascribable to binding of the Fcγ portion of ICOS-Ig to DC Fcγ receptors, since no effect was displayed by recombinant Fcγ fragments, CTLA-4-Ig, or total human IgG.

The effect on IL-1β and IL-23 was particularly striking since both cytokines were minimally induced by LPS alone, and B7h costimulation greatly increased their secretion. IL-1β is a leaderless proteinlacking a secretory signal sequence, and is released through a nonclassical pathway that avoids the ERGolgi route[35,36]. It is synthesized as a 35 kDa inactive precursor (pro-IL-1β) and secreted in the 17 kDa mature bioactive form upon cleavage by caspase-1, alias IL-1β-converting enzyme (ICE). In macrophages, induction of IL-1β secretion requires two signals[31,32]. The first senses microbe invaders and is delivered by macrophage pattern recognition receptors (PRR) binding pathogen-

associated molecular pattern molecules (PAMP) expressed by the invader, such as Toll Like Receptor 4 (TLR4) binding LPS. The second signal senses tissue injury and can be delivered by components of the P2 receptor (P2R) family binding nucleotides (ATP, UTP, ADP, UDP) released by dying or injured cells.

The first signal induces synthesis of pro-IL-1β, most of which is stored in the cytosol, but a fraction moves into specialized secretory lysosomes, where it colocalizes with inactive procaspase-1. The second signal triggers conversion of the inactive procaspase-1 to active caspase-1 by inducing aggregation of a multimolecular complex called the "inflammasome". Activated caspase-1 processes pro-IL-1β to mature IL-1β, which is then secreted. In the absence of P2R triggering, IL-1β secretion is minimal.

DC express several PRR, including TLR4, and two P2R, i.e. P2YR and P2XR, but they are weakly effective in inducing IL-1β secretion, which suggests that other second signals are required in these cells[37]. Our data show that such a function may be exerted by B7h reverse signalling, since it triggers secretion of high amounts of IL-1β in LPS-stimulated DC. In line with this view, LPS induced pro-IL-1β synthesis, but not caspase-1 activation, whereas B7h triggering induced caspase-1 activation, but not pro-IL-1β synthesis. Each stimulus was therefore insufficient for secretion of active IL-1β, whereas this was induced at high levels by both stimuli. The inflammasome-mediated effect displayed by B7h reverse signalling was confirmed by the observation that IL-1β release was blocked by inhibition of either caspase-1 by YVAD, or K+ efflux by KCl. A peculiarity is that, in macrophages, IL-1β secretion generally parallels secretion of IL-18, which is also processed by the inflammasome, but we did not detect any IL-18 secretion in our DC, possibly because LPS did not induce IL-18 synthesis[38].

Since IL-1β is envisaged as a proinflammatory cytokine, the effect of B7h on its secretion was in apparent contrast with the anti-inflammatory effect that would depend on the IL-10 increase and TNF-α decrease induced by B7h costimulation. This suggests that the cytokine milieu induced by B7h triggering may be tuned to elicit some peculiar response. IL-1β and TNF-α are envisaged as prototypic proinflammatory cytokines because of their ability to activate macrophages and vascular endothelial cells, induce liver release of acute phase mediators, and act as endogenous pyrogens, but they also display several substantial functional differences. In particular, IL-1β has several effects on T cell activation[31] that are not shared with TNF-α, and has recently been shown to play a key role in differentiation of Th17 cells, which constitute a new subset of proinflammatory Th cells characterized by secretion of IL-17A, IL-17F, IL-21, IL-22, and IL-26 (in humans)[34].

Th17 differentiation is driven by several cytokines acting in the absence of Th1 and Th2 polarizing conditions, and involves the RORc and RORa transcription factors. Mouse Th17 cell differentiation is driven by TGF-β1 and IL-6, and is autocrinally amplified by IL-21, whereas IL-23 seems to support expansion of differentiated Th17 cells[34]. In humans, Th17 differentiation is driven by IL-1β, IL-6, IL-23, and the role of TGF-β1 is still debated [39-44]. The fact that DCLPS/ICOS strikingly upregulated both IL-1β and IL-23 strongly suggests that they were tuned to elicit Th17 cell responses. In line with this possibility, they elicited secretion of high levels of IL-17 and IL-22, and low levels of IFN-γ, which is known to inhibit Th17 cell differentiation, in allogeneic lymphocytes.

The ability of DCLPS/ICOS to elicit IL-17 secretion was ascribable to IL-1β, since it was inhibited by anti-IL-1β antibodies; moreover, addition of exogenous IL-1β enabled DCLPS to elicit IL-17 secretion.

Intriguingly, this ability also involved triggering of ICOS on the T cell surface, since blockade of the B7h:ICOS interaction inhibited IL-17 secretion in the MLC system. In line with a role played by ICOS triggering, we showed that ICOS-mediated costimulation promoted differentiation of naïve Th cells toward Th17 cells, secreting IL-17 and IL-22, and expressing RORc and RORa, provided that Th17-polarizing cytokines, i.e. IL-1β and especially IL-1β+IL-6, were added to the cultures.

In previous studies, we had shown that ICOS triggering has striking polarizing effects on activation of human naïve T cells. These depended on the cytokine milieu since it induced striking IFN-γ secretion in the presence of IL-2, whereas it induced IL-10 and TGF-β in its absence. The present work refines this model and shows that ICOS triggering is a potent stimulator of Th17 differentiation in the presence of IL-1β, whose secretion, in turn, is strikingly induced in DC by ICOS-mediated triggering of B7h. Therefore, the ICOS:B7h interaction between DC and T cells triggers bidirectional signals that closely cooperate to induce differentiation of Th17 cells. This bidirectional signalling seems to be different from that induced by the CD28:B7.1/2 interaction, since B7.1/2 triggering did not induce IL1-β secretion in DC, and CD28 triggering was strikingly less effective in inducing Th17 cell differentiation from naïve Th cells[5].

METHODS

Cells. PBMC were obtained by density gradient centrifugation from buffy coats provided by the local Blood Transfusion Service (Novara, Italy).

iDC were prepared from CD14+ monocytes, isolated from PBMC with the Monocyte Isolation Kit II (Miltenyi Biotec), and culture for 5 days in culture medium, composed of RPMI 1640 (Invitrogen), 2mM L-glutamine (Invitrogen), and 10% fetal bovine serum (FBS) (Invitrogen), supplemented with recombinant human GM-CSF (800 U/ml) and IL-4 (10 ng/ml) (PeproTech).

In the maturation assays, iDC were cultured for 2 additional days in culture medium in the presence of LPS 1 μg/ml; Escherichia coli, serotype 055:B5, Sigma Chemicals Co.), or ICOS-Ig 1 μg/ml, or both.ICOS-Ig was a fusion protein of the extracellular portion of human ICOS fused to the human IgG1 Fc (R&D Systems); the soluble IgG1 Fc was used as a control (R&D Systems) and had no effect on DC maturation; in some experiments, a control was performed with CTLA-4-Ig 1 μg/ml (Immunotools) or human IgG whole molecule (Rockland). For caspase-1 inhibition, and to block K+ efflux, 50 μM YVAD (Alexis Biochemicals) and 160 mM KCl (Sigma) were respectively added to the culture.

The DC surface phenotype was assessed by immunofluorescence and flow cytometry using FITC- and PE-conjugated mAb to CD14, HLA-DR (Caltag), CD1a, CD80, CD83, CD86, HLA-A,B,C (Becton Dickinson), and B7h (eBioscience).

Naïve CD4+ T cells were purified by panning to remove CD11b+, CD45RO+ and HLA-DR+ cells with the appropriate mAbs, followed by use of the CD4+ T Cell Isolation Kit II (Miltenyi Biotec). This approach provided >98% cells displaying the phenotype CD3+CD4+CD45RA+CD14-CD16-CD19-CD20-, as assessed by direct immunofluorescence and flow cytometry.

Mannose receptor-mediated endocytosis. Cell endocytosis was assessed by evaluating uptake of FITC-dextran (molecular weight, 70 kDa; Molecular Probes)45. Briefly, each DC sample was split into two fractions of 1 x 105 cells which were incubated with 1 mg/ml FITC-dextran at either 37° or 0° C for 60 min. Uptake was then stopped by adding ice-cold PBS followed by extensive washes in a refrigerated centrifuge, and cells were analysed by flow cytometry.

Mixed leukocyte culture (MLC). DC (5x103) were co-cultured in 96-well round-bottom plates with allogeneic monocyte-depleted PBL for 5 days at a 1:20 DC:lymphocyte ratio. Supernatants were then collected and used for cytokine analysis. In the final 6 h of culture, 0.5 μCi [3H] thymidine (Amersham) were added to each well; cells were then harvested with a semiautomatic cell harvester and their radioactivity was measured with a β-counter (Perkin Elmer).

Analysis of the IL-1β secretion pathway. iDC (106/ml) prepared as described above were cultured with the maturation stimuli for 30 h in RPMI 1640 supplemented with 1% Nutridoma-HU (Boheringer) at 37°C. Culture supernatants and cells were then collected: the former for ELISA detection of the active form of IL-1β (see below) and Western blot analysis of caspase-1; the latter for western blot analysis of pro-IL-1β. Proteins from supernatants were precipitated with 20% trichloroacetic acid, resuspended in SDS-PAGE Loading Buffer (63 mM Tris-HCl pH 6.8, 10%, Glycerol, 2% SDS, 0.02% bromophenol-blue) whereas cells were lysed for 20 min on ice in AKT buffer (10 mM NaCl, 10 mM MgCl2, 10 mM Tris-HCl pH 7.5, 1% Triton X-100, 1% sodium deoxycholate, Aprotinin 1 µg/ml, Leupeptin 1 µg/ml, Pepstatin A 1 µg/ml, PMSF 100 µg/ml), and cleared by 20 min centrifugation at 13000 rpm at 4°C. Proteins were separated on 10% or 8-15% SDSPAGE gels, and transferred to nitrocellulose. Filters were then blocked in TBS (10 mM TrisHCl pH 7,9; 150 mM NaCl.) containing 5% nonfat milk and 1% Tween 20 for 1 h, and incubated for 16 h with anti-IL-1β mAb (1 µg/ml; 2805, R&D Systems) or rabbit anti-human caspase-1 polyclonal antibody (Alexis Biochemicals), followed by the appropriate horseradish peroxidase-conjugated secondary reagent (Dako Cytomation), and detected by enhanced chemiluminescence.

Th cell differentiation assay. Round-bottom 96-well plates were coated with 100 µl of anti-CD3 mAb (10 µg/ml, OKT3) overnight at 4°C. To stimulate ICOS or CD28, plates were washed with PBS and further coated with the indicated concentrations of human B7h-Ig (5 µg/ml; R&D System), B7.1-Ig (5µg/mL; R&D System) or polyclonal anti-mouse antibody (10 µg/ml; Dako Cytomation) followed by anti-CD28 mAb (5 µg/ml; ANC28.1/5D10; Ancell) at room temperature for 2 hours. Plates were then washed with PBS and purified naïve CD4+ T cells were seeded at 105 cells/well in triplicate in 200 µl of in RPMI 1640 plus 10% FBS in the presence of neutralizing anti-IFNγ (4 µg/ml; 25718; R&D Systems) and anti-IL4 (2 µg/ml; 34019; R&D Systems) antibodies, in the presence and absence of human IL-1β (10 ng/ml; PeproTech) and IL-6 (20 ng/ml; PeproTech). To analyze cytokines, supernatants were collected at day 5 of culture or at different time as shown in the kinetic experiments, whereas cells were collected for RNA extraction and real time evaluation. Proliferation was performed in the same conditions described for MLC.

Cytokine analysis. In the DC supernatants, IL-1β, IL-6, IL-10, IL-12p70, and TNF-α were evaluated with a cytofluorimetric bead assay (Human Inflammation CBA kit, Becton Dickinson). In the MLC supernatants, IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ were evaluated with the Human Th1/Th2 CBA kit (Becton Dickinson). Standard ELISA were also

used to evaluate secretion of IL-17A, IL-22, TGF-β1 or the 17KDa active form of IL-1β (R&D Systems) and IL-23 (eBioscience).

Real-time quantitative RT-PCR. Total RNA was extracted with TRIzol (Sigma). RNA (500 ng) was retrotranscribed by the ThermoScriptTM RT PCR System (Invitrogen) by random hexamers.

Transcripts were quantified by real-time quantitative PCR on an ABI PRISM 7000 sequence detector (Applied Biosystems) with Applied Biosystems predesigned TaqMan Gene Expression Assays in triplicate for each sample. The following probes were used (Applied Biosystems assay identification numbers in parentheses): RORc (Hs01076112_m1), RORa (Hs00931151_m1). For each sample, mRNA abundance was normalized to the amount of ribosomal protein hypoxanthine-phosphoribosyltransferase (HPRT- Hs99999909_m1) or Glyceraldehyde 3-phosphate dehydrogenase APDH (Hs99999905_m1), as indicated.

Statistical analysis. The non-parametric paired Wilcoxon test was used to compare differences in all experiments. Values of P values of 0.05 or less were considered statistically significant.

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Table 1. Expression of activation/maturation markers by DC treated with different stimuli

cell type[a]

	cell type[a]			
	DCCTR	**DCICOS**	**DCLPS**	**DCLPS/ICOS**
B7.1	7.4[b] ± 0.9	21.6 ± 2.1*	119.17 ± 16.2*	95.5 ± 13.1*
B7.2	34.9 ± 6.3	25.6 ± 3.7*	531.26 ± 38.8*	517.7 ± 31.5*
B7h	6.8 ± 0.6	7.2 ± 0.57	6.54 ± 0.5	6.37 ± 0.5
HLA-ABC	75.5 ± 4.8	71.5 ± 6.29	132.7 ± 10.9*	169.4 ± 10.9*
HLA-DR	246.3 ± 15.2	323.3 ± 19.6*	353.9 ± 11.6*	268.9 ± 25.2**§**
CD83	3.1 ± 0.3	7.2 ± 0.57*	15.1 ± 2.3*	18.4 ± 2.5*

[a] iDC were cultured for 2 days in complete medium alone (DCCTR) or ICOS-Ig (DCICOS), LPS (DCLPS), or LPS+ICOS (DCLPS/ICOS). Cells were stained with the appropriate mAb and analyzed by flow cytometry.

[b] Results are expressed as mean fluorescence intensity (MFI) in arbitrary units and are the mean ± SE from 8 independent experiments. Statistical analysis was performed using the Wilcoxon test for paired samples (*: P<0.05 compared with DCCTR; §: P<0.05 compared with DCLPS).

FIGURE LEGENDS

Figure 1. Characterization of iDC and their response to LPS and B7h triggering. a) Flow cytometry of monocytes and iDC stained with mAb to CD14, CD1a, B7.1, B7.2 and B7h. iDC were analyzed after 5 days of culture with GM-CSF and IL-4. Control staining was performed with isotypematched Ig. One experiment representative of eight is shown.

b) Endocytic activity of DC treated with different stimuli. B7h triggering does not influence the endocytic activity of either iDC or LPS-treated DC. iDC were treated with control medium (DCCTR), ICOS-Ig (DCICOS), LPS (DCLPS), and LPS+ICOS-Ig (DCLPS/ICOS) for two days and uptake of FITCDextran was then assessed by flow cytometry. Grey and white histograms show staining of cells incubated with FITC-Dextran at 37°C and 0°C (negative control) respectively. Percentages of FITCpositive cells are indicated. One experiment representative of six is shown.c) Induction of allogeneic lymphocyte proliferation by DC treated with different stimuli. B7h triggering substantially inhibits the allostimulatory activity induced by LPS. DCCTR, DCLPS, DCICOS, and DCLPS/ICOS were used as stimulators against allogeneic PBL at a stimulator/responder ratio of 1:20 for 5 days. [3H] thymidine (0.5 μCi/well) was added in the final 6 h of culture and radioactivity uptake was measured by a β-counter. All experiments were performed in triplicate. Results are expressed as mean ± SE of the results from ten experiments. Statistical analysis was performed using the Wilcoxon test for paired samples (*: P<0.05 compared to DCLPS).

Figure 2. Cytokine secretion by DC treated with different stimuli. a) B7h triggering substantially modulates the cytokine pattern secreted by DC. iDC were stimulated with LPS, ICOS-Ig, or LPS plus ICOS-Ig and culture supernatants were harvested after 2 days and examined for cytokine production.

Data represent the means ± SE of results from six independent experiments. Statistical analysis was performed using the Wilcoxon test for paired samples (*: P<0.05 compared

with DCCTR; §: P<0.05compared with DCLPS). Note that different scales were used for different cytokines.

b) Kinetic experiments showing IL-1β and IL-23 secretion at different times after stimulation with LPS or ICOS-Ig. One experiment representative of three is shown.

Figure 3. Analysis of IL-1β and caspase-1 in DC stimulated with different stimuli. a) iDC were cultured for 30 h in the presence of medium (DCCTR), LPS (DCLPS), ICOS-Ig (DCICOS) or LPS+ICOSIg (DCLPS/ICOS). Then, cell lysates were analysed for pro-IL-1β expression by Western blot (upper panel), and cell supernatants for secretion of active caspase-1 by Western blot (middle panel) and active IL-1β by ELISA (lower panel). The antibodies to IL-1β and caspase-1 used in the Western blot experiments did not discriminate between the non-active and active forms of these molecules, but discrimination was obtained by molecular size. By contrast, IL-1β detection was performed in the supernatants using an ELISA specific for the active form of IL-1β. One experiment representative of six is shown.

b, c) ELISA evaluation of active IL-1β secretion by iDC stimulated with b) LPS+ICOS-Ig in the presence and absence of the caspase-1 inhibitor YVAD (50 μM) or KCl (160 mM) inhibiting the K+ efflux; c) LPS+ICOS-Ig, or +IgG1-Fc, or +CTLA-4-Ig, or + total IgG. One experiment representative

of three is shown.

Figure 4. Cytokine secretion in allogeneic MLC stimulated by DCLPS and DCLPS/ICOS. a) B7h triggering substantially modulates the cytokine pattern induced by LPS-stimulated DC in MLC. DCLPS, and DCLPS/ICOS were used as stimulators in MLC with allogeneic PBL. After a 5-day culture, cytokine secretion was assessed in the supernatants. Data represent the means ± SE of the results form five independent experiments. Statistical analysis was performed using the Wilcoxon test for paired samples (*: P<0.05).

b) Effect of exogenous IL-1β and anti-IL-1β antibodies on the ability of DCLPS and DCLPS/ICOS respectively to elicit IL-17 secretion in allogeneic MLC. Addition of IL-1β enables DCLPS capable to elicit IL-17 secretion, whereas anti-IL-1β antibodies inhibit the ability of DCLPS/ICOS to elicit IL-17 secretion. One experiment representative of three is shown.

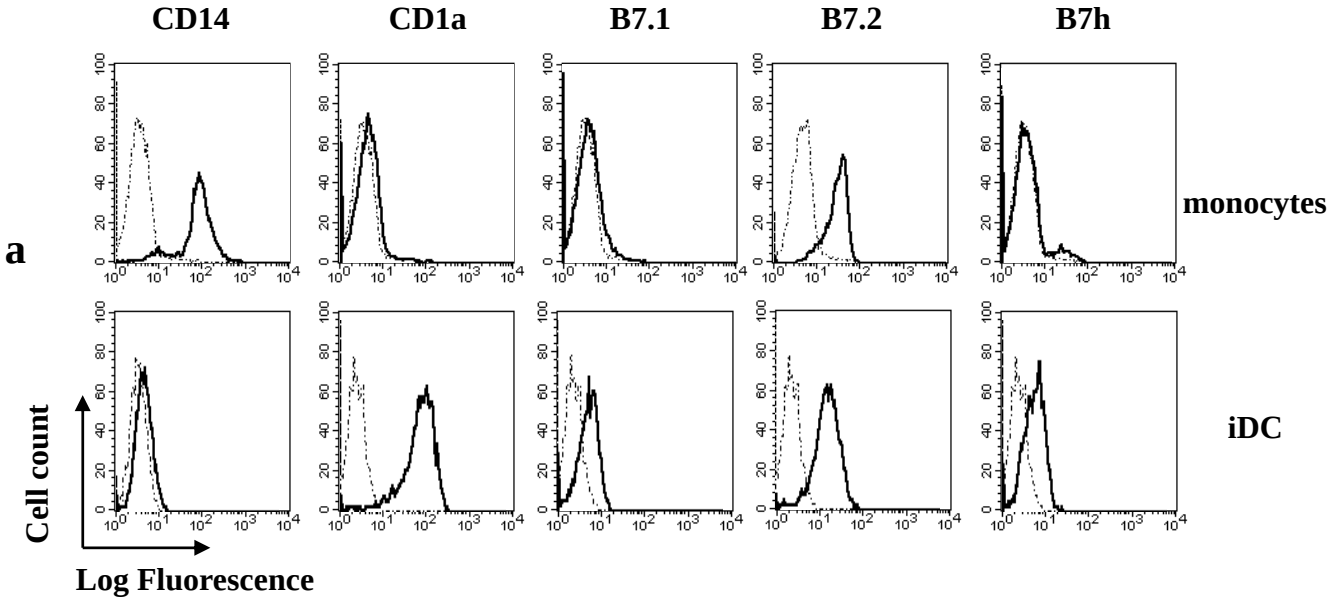
c) Effect of ICOS-Ig on the ability of DCLPS/ICOS to elicit IL-17 secretion in allogeneic MLC. ICOS-Ig was added to MLC at the start of culture, and IL-17 secretion was evaluated at day 5. One experiment representative of three is shown.

Figure 5. IL-17 and IL-22 secretion by naïve CD4+ T cells primed with different stimuli. Naïve CD4+ T cells were seeded in wells coated with anti-CD3 mAb in the presence and absence of B7h-Ig or B7.1-Ig or anti-CD28 mAb, and incubated in the presence and absence of exogenous IL-1β or IL-1β+IL-6, as indicated. At day 5, secretion of IL-17 was assessed by ELISA in the supernatants (a).

Data represent the means ± SE of the results from six independent experiments. Statistical analysis was performed using the Wilcoxon test for paired samples ($P<0.05$ *: significantly different from the same costimulus in the absence of exogenous cytokines; § significantly different from anti-CD28- and B7.1-Ig-driven costimulation in the same cytokine milieu). Note that different scales were used for different cytokines.

b) Kinetic experiments showing IL-17 and IL-22 secretion evaluated by ELISA in the supernatants at different times after treatment with the indicated stimuli. One experiment representative of three is shown.

c) Real time PCR analysis of the expression of RORc and RORa mRNA in naïve CD4+ T cells differentiated in vitro for 5 days in the presence of anti-CD3 mAb plus B7h-Ig and various cytokine combinations, as indicated. Cycling threshold values are normalized to those of mRNA encoding HPRT or GAPDH as shown. One experiment representative of four is shown.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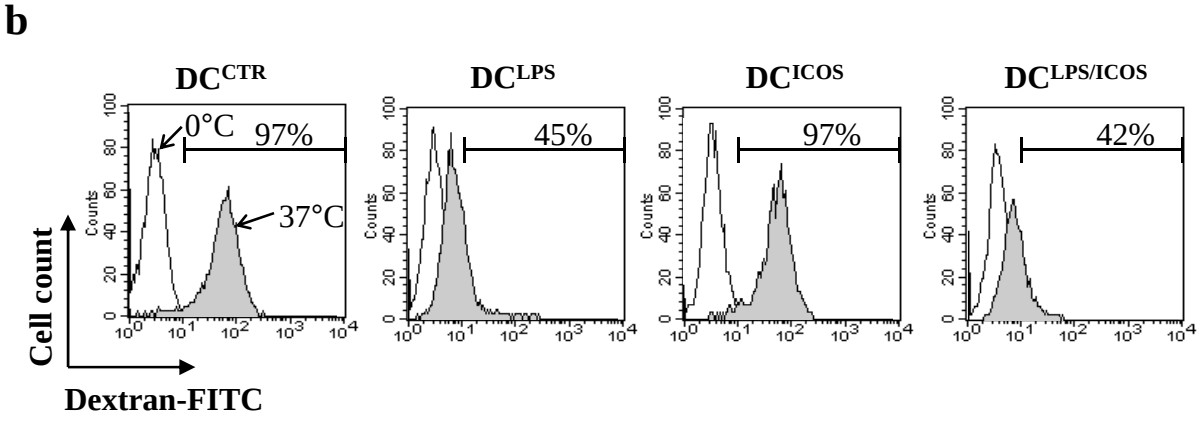
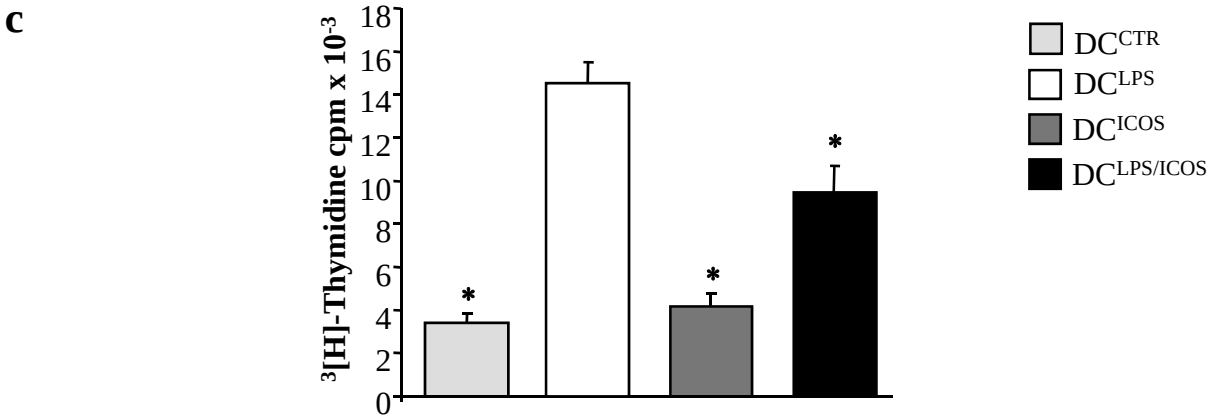
CD1a
B7.1
B7.2
B7h
monocytes
iDC
Cell count
Log Fluorescence


b
DC^CTR
DC^LPS
DC^ICOS
DC^LPS/ICOS
0°C
37°C
97%
45%
97%
42%
Counts
Cell count
Dextran-FITC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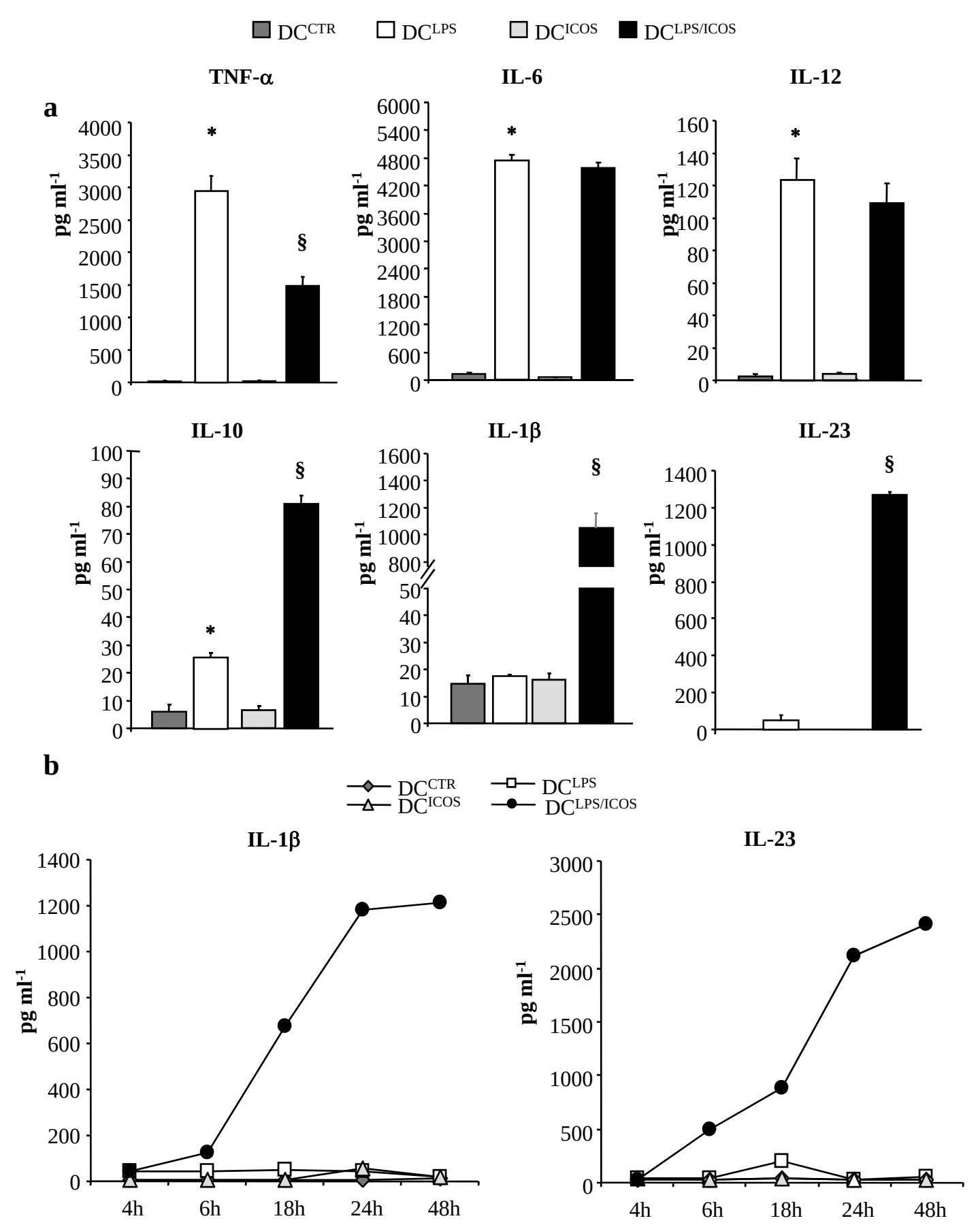
DC^CTR
DC^LPS
DC^ICOS
DC^LPS/ICOS

Figure 1


DC^CTR
DC^LPS
DC^ICOS
DC^LPS/ICOS
a
TNF-α
IL-6
IL-12
IL-10
IL-1β
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b
IL-1β
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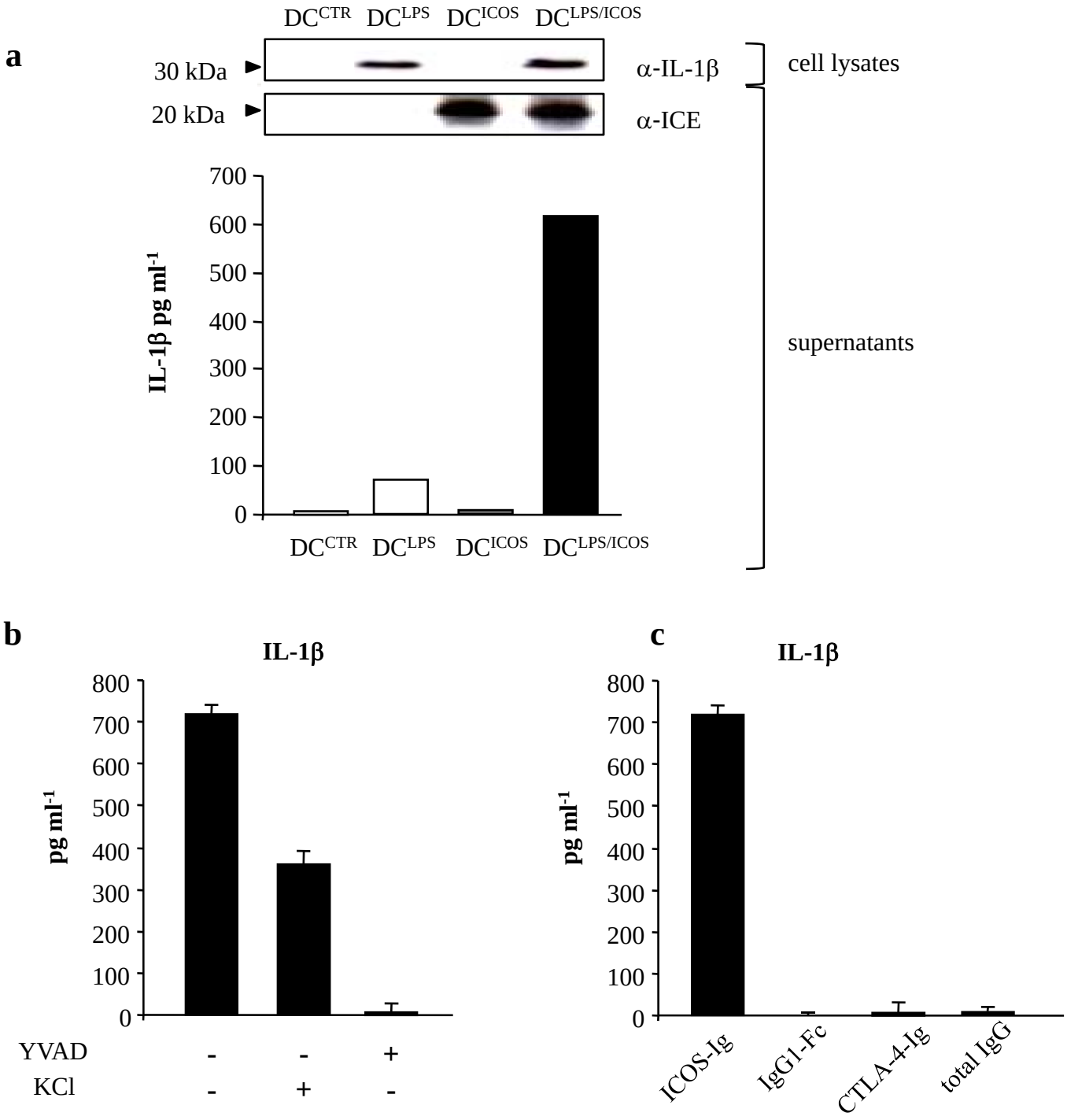
Figure 2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cell lysates
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700
600
500
400
300
200
100
0
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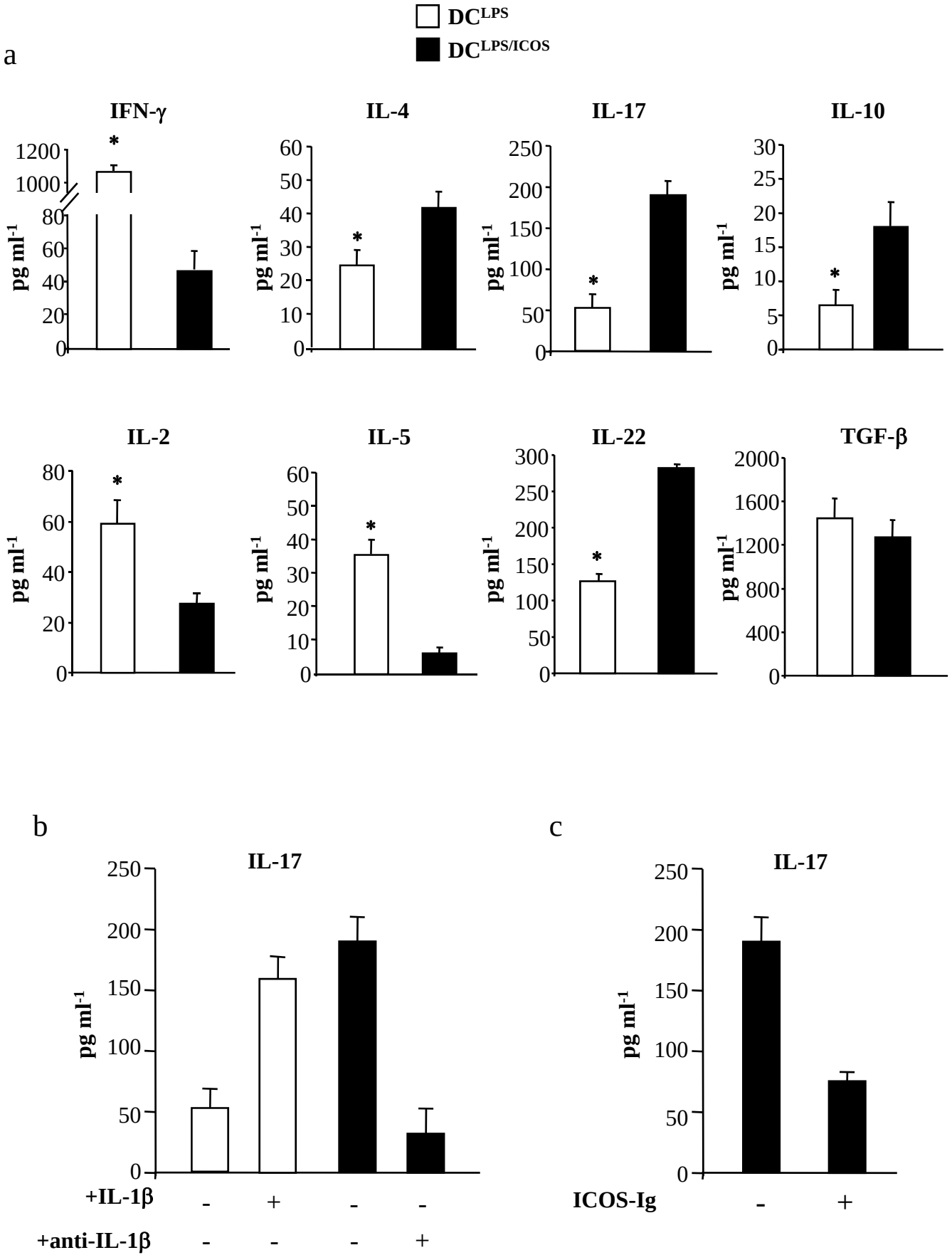
b
IL-1β
pg ml-1
800
700
600
500
400
300
200
100
0
YVAD - - +
KCl - + -
c
IL-1β
pg ml-1
ICOS-Ig
IgG1-Fc
CTLA-4-Ig
total IgG

Figure 3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IL-10
IL-2
IL-5
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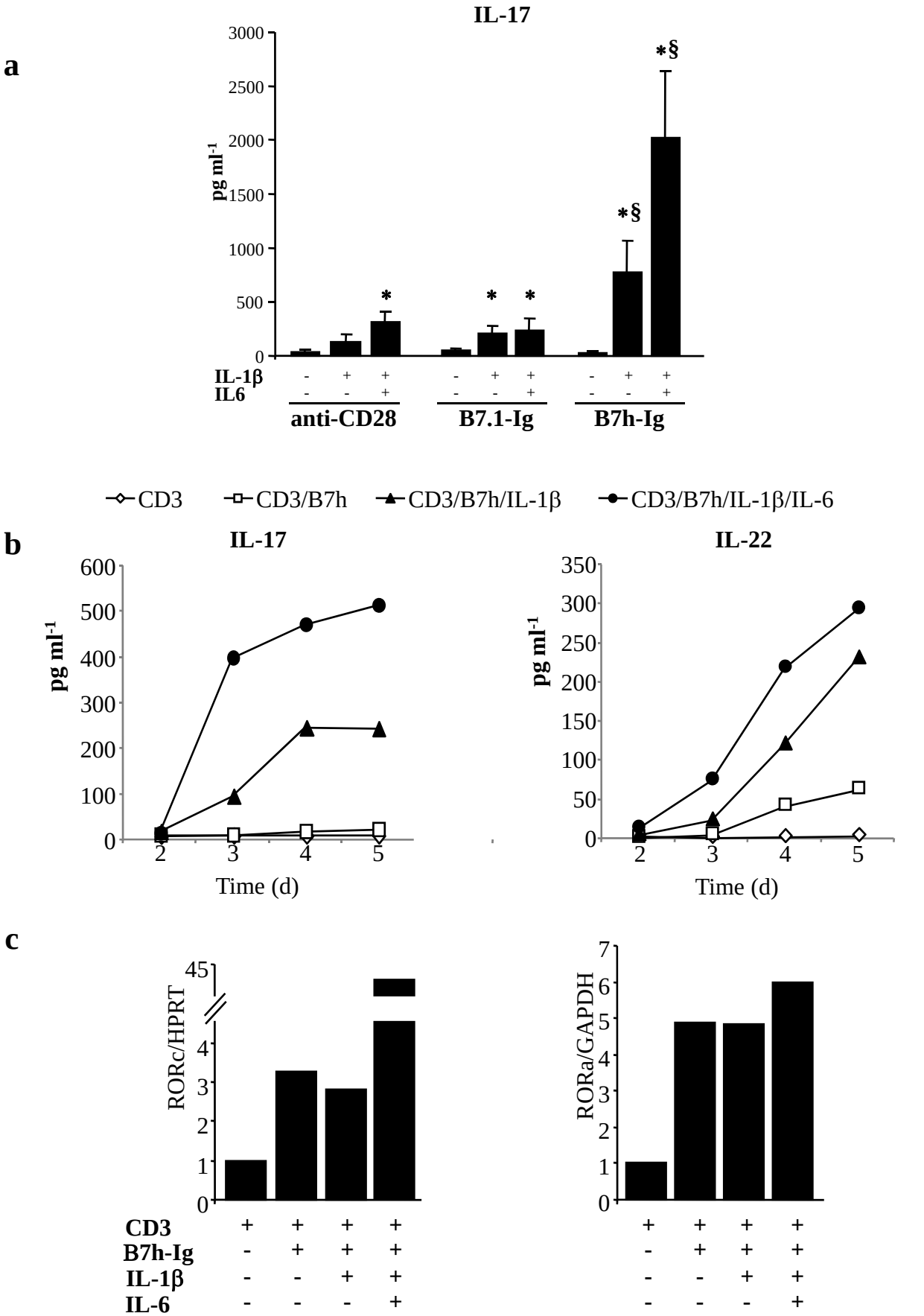
pg ml-1
b
IL-17
+IL-1β
+anti-IL-1β
c
IL-17
ICOS-Ig

Figure 4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2000
1500
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500
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IL6
anti-CD28
B7.1-Ig
B7h-Ig
CD3
CD3/B7h
CD3/B7h/IL-1β
CD3/B7h/IL-1β/IL-6
b
IL-17
IL-22
pg ml-1
Time (d)
c
RORc/HPRT
RORa/GAPDH
CD3
B7h-Ig
IL-1β
IL-6

Figure 5

Risultati (3)

EFFETTI DELL'INTERAZIONE ICOS/B7h IN UN MODELLO MURINO DI CARCINOMA MAMMARIO

Introduzione

Secondo la teoria dell'immunosorveglianza, il sistema immunitario specifico può riconoscere le cellule tumorali e opporsi allo sviluppo della neoplasia, riconoscendo antigeni tumore-specifici o tumore-associati. Un tumore è in grado di attivare una risposta da parte dei linfociti T se esprime in superficie molecole MHC. Perchè la riposta sia efficiente il linfocita deve però anche ricevere un segnale costimolatorio. Le molecole costimolatorie sono generalmente espresse solo su cellule immunitarie, con l'eccezione di B7h, che e' anche espresso su altri tipi cellulari, tra cui cellule endoteliali vascolari e cellule stromali attivate.

Il bilancio di espressione di molecole costimolatorie e coinibitorie sulla superficie delle cellule tumorali è fondamentale perchè queste possano indurre un'efficace risposta dei linfociti T (*tumor surveillance*). La mancata espressione di molecole costimolatorie rende il tumore invisibile al sistema immunitario, così come una aumentata espressione di molecole inibitorie.

Nonostante i modelli murini non sempre rispecchino esattamente ciò che succede nell'uomo sono comunque una base di partenza per condurre studi funzionali in un sistema più complesso rispetto a quello necessariamente limitato delle colture *in vitro*.

Per valutare il ruolo dell'interazione B7h-ICOS nell'immunosorveglianza anti-tumorale, abbiamo scelto di lavorare su un modello di tumore spontaneo nel topo.

Lo studio della relazione tra tumorigenesi e sistema immunitario è un campo in costante sviluppo e sempre più promettente: sembra infatti verosimile poter prevenire, inibire ed eradicare tumori attivando in maniera controllata e mirata i vari "attori" della risposta immunitaria e un *target* particolarmente interessante sembrano essere proprio le molecole costimolatorie.

Il modello murino utilizzato, BALB-neuT [Pannellini et al., 2004], è *knock-in* per un gene coinvolto nello sviluppo del tumore mammario (HER2/neu) e permette di lavorare su topi femmina che invariabilmente sviluppano tumori a tutte le ghiandole mammarie con una cinetica e una stadiazione fisse e molto simili a quelle riscontrate nella donna.

Ciò ha permesso quindi di lavorare su un sistema complesso, ma controllato e di sviluppare il progetto su due fronti: un primo *screening* è stato eseguito trattando femmine BALB-neuT con un anticorpo anti-ICOS per valutare se questo influenzasse la cinetica di sviluppo dei tumori in confronto a femmine non trattate. In parallelo si stanno incrociando i topi BALB-neuT con topi ICOS KO per studiare lo sviluppo di tumori in una progenie *inbred*.

Nel nostro modello sperimentale, la stimolazione di ICOS con l'anticorpo C398.4 A si è dimostrata efficace nel ritardare lo sviluppo dei tumori mammari in topoline BALB-neuT.

Materiali e metodi

1. *Strains murini utilizzati*

1.1 BALB/c

Lo *strain* BALB/c (Fig 5) fu creato da McDowell nel 1923. E' tipicamente usato in molti protocolli sperimentali e soprattutto nella produzione di anticorpi monoclonali. E' *inbred,* albino e di piccola taglia.

Le principali caratteristiche sono:

- Bassa incidenza di tumori mammari
- Resistenza ad arterosclerosi indotta dalla dieta.
- Alta incidenza di difetti cardiaci
- Sensibile alle radiazioni

Fig 5 – BALB/c

Nei miei esperimenti ho utilizzato la variante BALB-neuT descritta da Pannellini et al, 2004.

2. Genotipizzazione

I topi BALB-neuT vengono ottenuti per incrocio di femmine BALB/c con maschi BALB-neuT, che sono eterozigoti per Her2/neu. Quindi la prole deve essere genotipizzata perchè alcuni topi saranno neu -/- e altri neu+/-.

La genotipizzazione si effettua per amplificazione mediante PCR su DNA estratto da frammenti di coda.

2.1 Estrazione del DNA da coda

Si tagliano 1-2 mm di coda all'animale: per solubilizzare la cheratina e liberare il DNA si incuba ogni campione con 200 μl di NaOH 50mM per 10 minuti a 95°C, in agitazione.

Quindi si neutralizza con Tris/HCl 1M pH 8 (50 μl) e si agita con vortex per circa 1 minuto e si centrifuga a 13000 rpm per 6 minuti. A questo punto si recupera il surnatante e lo si conserva a 4°C. Si quantizza il DNA estratto con nanodrop e si utlizza direttamente per amplificare con PCR o si conserva a 4°C.

2.2 Amplificazione del DNA

Le condizioni di PCR, il ciclo e i primer utilizzati sono indicati nelle tabelle che seguono. (Tab 1, 2, 3, 4)

bp	Sqz 5'-3'	OLIGO	T_m (°c)	$T_{annealing(°c)}$
265	GACTGCAACTGCTCCTGGC	**ICOS fw**	60	56
	CATTGGATTCTTGATGGACAC	**ICOS3 rev**	66	62
478	ATTGAACAAGATGGATTGCAC	**neo fw**	58	54
	TCTTCGTCCAGATCATCCT	**neo rev**	56	52
230	GTAACACAGGCAGATGTAGGA	**neuT fw**	62	58
	ATCGGTGATGTCGGCGATAT	**neuT rev**	60	56
~300	TTCTTGGGTATGGAATCCTG	**b-actina fw**	58	54
	CTAGAAGCACTTGCGGTGCA	**b-actina rev**	62	58

Tab. 1 – Primers utilizzati

1) BALB-neuT

REAGENTS	FINAL CONC	μl(final 25)
H2O		0,4
buffer (10X)	1X	2,5
$MgCl_2$ (25mM)	1,5 mM	**1,5**
dNTP (2mM)	0,2 mM	2,5
neuT fw	0,5 μM	2,5
neuT rev	0,5 μM	2,5
b-actina fw	0,5 μM	2,5
b-actina rev	0,5 μM	2,5
Taq (5U/μl)	* 0,5 U/reaction	0,1
DNA sample	2,5 μl	8
		25

Tab 2 – Concentrazione dei reagenti utilizzati per amplificare il gene Her2/neu e il controllo positivo β-actina

Il tipico profilo che si vede correndo su gel di agarosio al 3% i prodotti della PCR è il seguente (fig 6):

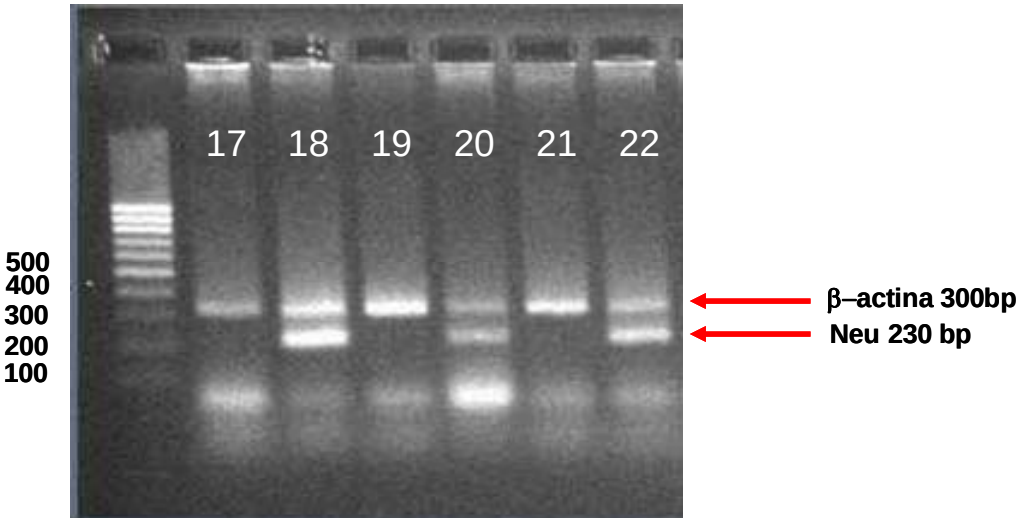

Fig 6 – gel di agarosio al 3%, prodotti di PCR con primer neu e beta-actina

3. Estrazione di cellule della milza

La milza dell'animale viene estratta in condizioni di sterilità e le cellule vengono portate in sospensione in terreno RPMI 10%FBS (Gibco).

Le emazie vengono lisate mediante shock osmotico con acqua (900 μl) e successiva neutralizzazione con PBS 10X (100 μl).

Quindi si eliminano i detriti cellulari mediante filtrazione e si contano le cellule. Mediamente da una milza si ottengono circa 90 milioni di cellule totali.

4. Analisi citofluorimetriche

Le analisi citofluorimetriche sono state effettuate su cellule totali estratte da milza.

Dopo un prima incubazione di 30 minuti con siero di ratto al 10% per bloccare i recettori Fc, le cellule sono state lavate e risospese in staining buffer al 10% in siero di ratto e colorate con i seguenti anticorpi:

- anti-ICOS - FITC
- anti-CD4-APC (BD Pharmingen)
- NK 1.1 – PE (e-Bioscience)
- anti- CD8-biotina (TIB105) + streptavidina-AlexaFluor647 (Molecular Probes)
- anti-CD3 – DyLi649
- anti-CD3γδ - FITC (BD Pharmingen)
- anti-CD25-biotina (BD Phermingen)+ streptavidina- AlexaFluor647 (Molecular Probes)
- anti-CD4 – FITC (GK 1.5)

- anti-FoxP3 – PE

La tinzione intracellulare di FoxP3 è stata effettuata mediante kit e-Bioscience (PE anti-mouse/rat FoxP3 staining set).

5. Trattamenti

Le femmine BALB-neuT sono state trattate con anticorpo anti-ICOS C398.4A (Buonfiglio et al., 2000), prodotto in hamster e attivo su cellule murine e umane.

L'anticorpo è stato iniettato per via intraperitoneale una volta a settimana ad una dose di 100μg/topo per 4 settimane seguite da settimane di pausa. Il ciclo viene ripetuto per tutta la vita dell'animale. Come controllo sono state utilizzate immunoglobuline di criceto (*normal hamster* Ig).

Risultati

La cinetica di sviluppo dei tumori nel modello murino BALB-neuT si studia misurando settimanalmente le dimensioni e il numero dei tumori nei topi femmina oggetto dell'esperimento (es. topi trattati vs. controlli; confronto tra topi con KO o KI vs controlli etc.). I dati raccolti permettono di confrontare:

- Settimana di comparsa del primo tumore
- Dimensioni dei tumori in relazione al numero di tumori per topo o al numero totale di ghiandole mammarie, cioè 10.
- Molteplicità tumorale (*Tumor multiplicity*)
- Settimana del sacrificio
- Percentuale di topi liberi da tumori (% *tumor free mice*)

In particolare femmine BALB-neuT sono state trattate con un anticorpo anti-ICOS (C398.4A) per valutare se questo influenzasse la cinetica di sviluppo dei tumori in confronto a femmine non trattate**.**

Effetto dell'anticorpo anti-ICOS C398.4A sullo sviluppo di tumori mammari in topi BALB-neuT femmina

Protocollo sperimentale: sono stati considerati 9 gruppi sperimentali di femmine BALB-neuT. Il trattamento è stato iniziato alla settima/ottava settimana (w7-8) di vita in 7 esperimenti e alla dodicesima (w12) in 2 esperimenti per vedere se il momento di inizio del trattamento potesse avere un'influenza sull'efficacia dello stesso. Istologicamente la differenza risiede nel fatto che alla w7-8 i tumori non si sono ancora sviluppati, mentre alla w12 sono quasi palpabili. Ogni gruppo sperimentale è stato suddiviso in 3 sottogruppi, sottoposti a 3 trattamenti differenti:

A) iniezione i.p. di C398.4A (4mg/Kg ovvero 100μg/topo) una volta alla settimana per 4 settimane, seguita da una pausa di 3 settimane.
B) iniezione i.p. di NHIg (*Normal Hamster Immunoglobulines*, controllo) (4mg/Kg ovvero 100μg/topo) una volta alla settimana per 4 settimane, seguita da una pausa di 3 settimane.
C) iniezione di PBS (200μl/topo, controllo) una volta alla settimana per 4 settimane, seguita da una pausa di 3 settimane.

Una volta alla settimana sono stati misurati numero e dimensione dei tumori. I primi tumori palpabili hanno cominciato a comparire alla settimana 14 (w14) di vita dell'animale. I topi sono stati sacrificati per ragioni etiche quando le dimensioni dei tumori superavano i 10 mm o quando tutte le ghiandole mammarie risultavano interessate, tra la w20 e la w27.

La somministrazione i.p. di C398.4A si è dimostrata efficace nel ritardare, seppure di poco (1 settimana, $p < 0,05$), la comparsa del primo tumore palpabile, rispetto al trattamento con PBS e con NH-Ig, che hanno dato risultati paragonabili. Da notare che il ritardo nell'inizio del trattamento (w12) si rispecchia in una comparsa precoce dei tumori: i topi trattati con C398.4A tendono a comportarsi come i controlli e il trattamento perde quindi la sua efficacia (Tab 5).

	w7-8		w12	
Trattamento	**Media**	**SE**	**Media**	**SE**
C398.4°	18,00	0,53	17,33	0,58
NH-Ig	16,92	0,40	16,40	0,16
PBS	16,17	0,41	16,83	0,53

Tab 5 – Settimana di comparsa del primo tumore (valori medi e mediane)

w7-8: trattamento iniziato alla settimana 7-8 di vita; w12: trattamento iniziato alla settimana 12 di vita. Iniezione i.p. una volta alla settimana (100μg/topo). $p < 0,01$ (t test di Student) confrontando topi trattati con C398.4 A sia con quelli trattati con PBS che con quelli trattati con NH-Ig. Dati mostrati come medie e SE

Nei 3 gruppi sperimentali non varia invece significativamente la durata media della vita dell'animale, ovvero l'età a cui è stato ritenuto opportuno il sacrificio (22 settimane).

Si è quindi seguito lo sviluppo nelle dimensioni dei tumori nei 3 gruppi in relazione al numero di tumori per topo (Fig. 7) o in relazione al numero di mammelle totali per topo e cioè 10 (Fig 8). In entrambi i casi le dimensioni dei tumori nei topi trattati con C398.4 A sono sensibilmente ($p < 0,01$) inferiori, a parità di settimana, rispetto a quelle dei topi di controllo.

Tale risultato è in linea con il ritardo nella comparsa del primo tumore precedentemente descritto: l'effetto del trattamento dell'anticorpo viene quindi mantenuto durante tutta la vita dell'animale.

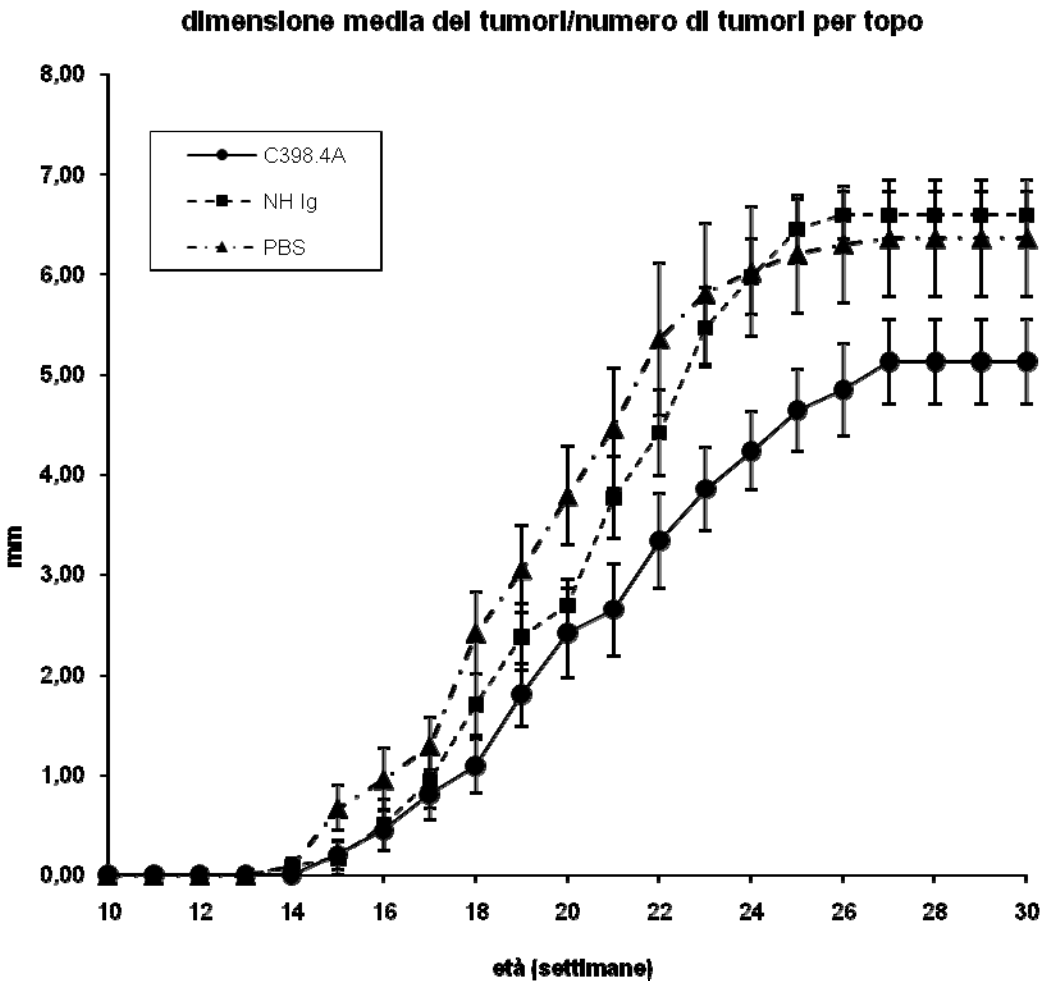

Fig 7 – Media delle dimensioni dei tumori in relazione al loro numero per singolo topo.

$p < 0,01$ (t test di Student) confrontando topi trattati con C398.4 A sia con quelli trattati con PBS che con quelli trattati con NH-Ig

Dati mostrati come medie e SE

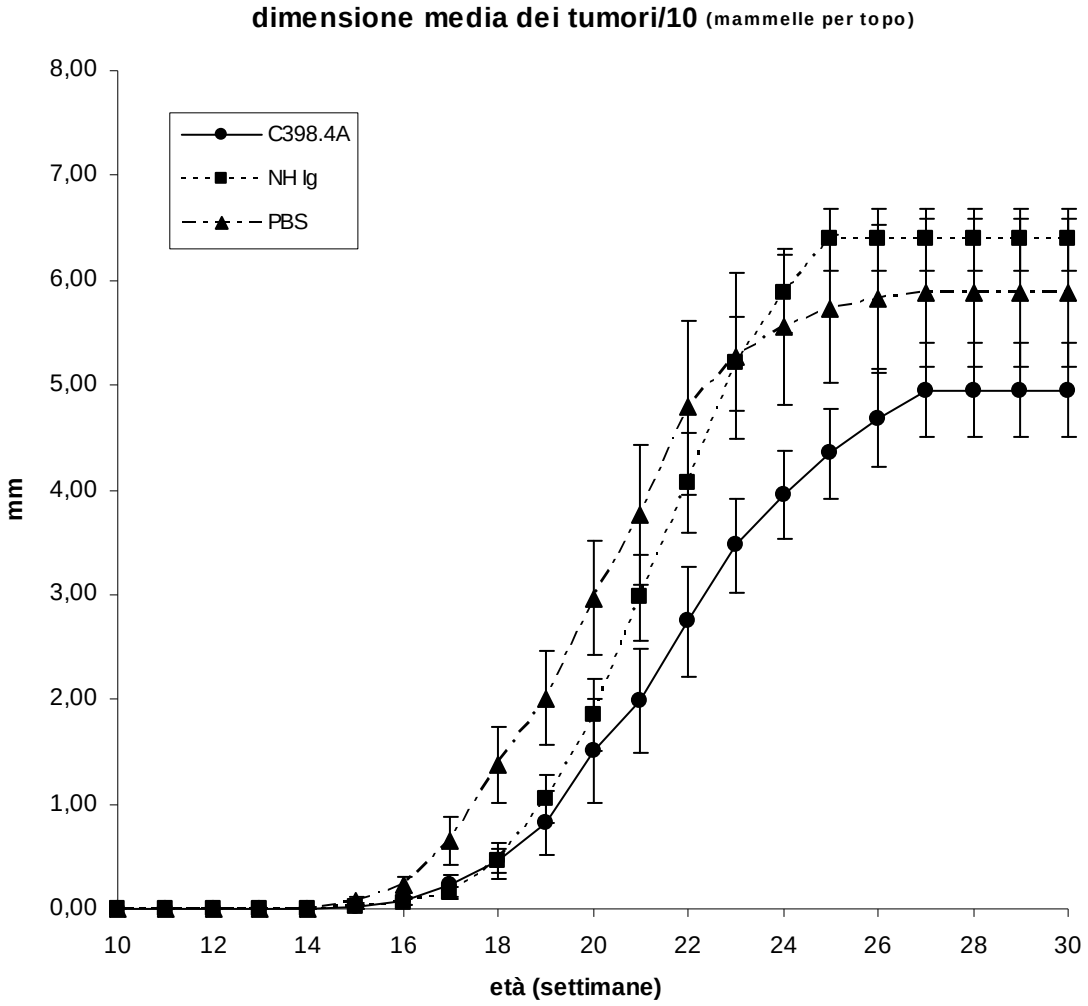

Fig 8 – Media delle dimensioni dei tumori in relazione al numero di ghiandole mammarie per topo, ovvero 10.
$p < 0,01$ (t test di Student) confrontando topi trattati con C398.4 A sia con quelli trattati con PBS che con quelli trattati con NH-Ig
Dati mostrati come medie e SE

Un *trend* leggermente differente si osserva se invece delle dimensioni si considera la molteplicità tumorale (*tumor multiplicity)*, ovvero il numero di tumori per topo, e la percentuale di topi liberi da tumori (*tumor free mice*): tra la settimana 17 e la settimana 23 di vita, i topi trattati con C398.4 A hanno sviluppato un numero di tumori inferiore rispetto ai controlli. La differenza più consistente si registra alla settimana 20 (4.73 vs 6.42~7.33) (Fig 9). Dalla settimana 23 la differenza comincia a scomparire perché anche nei topi trattati con C398.4 A vengono coinvolte tutte le 10 ghiandole mammarie, anche se da tumori di dimensioni inferiori rispetto ai controlli.

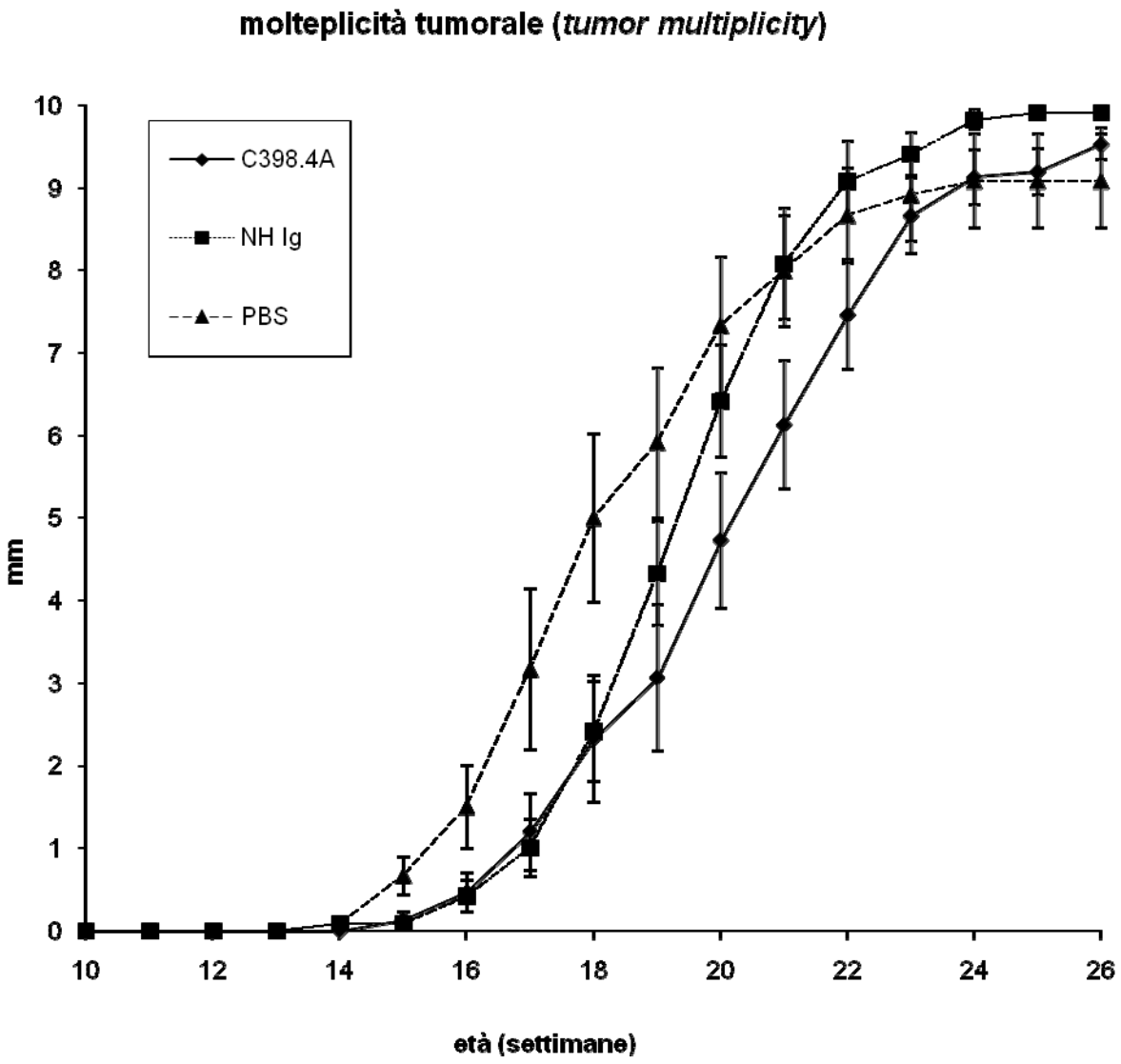

Fig 9 – Molteplicità tumorale (*tumor multiplicity),* ovvero numero di tumori per topo a parità di settimane di vita.
p < 0,01 (t test di Student) confrontando topi trattati con C398.4 A sia con quelli trattati con PBS che con quelli trattati con NH-Ig
Dati mostrati come medie e SE

Il ritardo nella comparsa di tumori precedentemente descritto nei topi trattati con C398.4A si rispecchia nella notevole differenza, tra la settimana 16 e 20 di vita, nella percentuale di topi liberi da tumore (Fig 10).

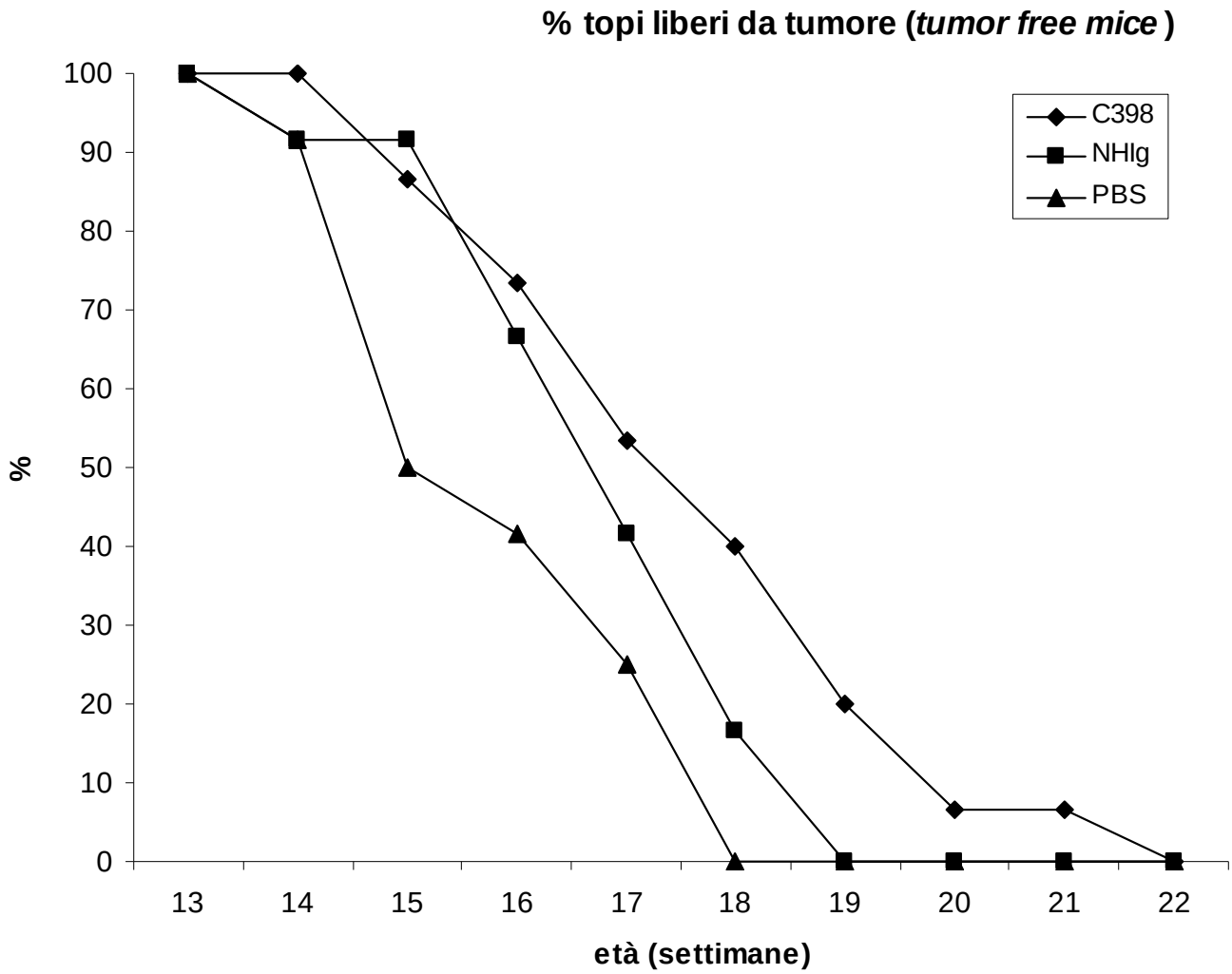

Fig 10 – Percentuale di topi liberi da tumore (*tumor free mice)*

p < 0,05 (t test di Student) confrontando topi trattati con C398.4 A sia con quelli trattati con PBS che con quelli trattati con NH-Ig

Sono state condotte analisi citofluorimetriche su cellule totali estratte da milza per valutare eventuali differenze tra topi trattati con C398.4A e controlli nell'espressione dei marcatori dei linfociti Treg (CD25 e FoxP3) ma non è stata notata nessuna differenza. Nemmeno la percentuale di NK (CD56+), T_H (CD4+) e T citotossici (CD8+) variava significativamente nei 3 gruppi sperimentali.

Discussione

Secondo la teoria dell'immunosorveglianza, il sistema immunitario specifico può riconoscere le cellule tumorali e opporsi allo sviluppo della neoplasia, riconoscendo antigeni tumore-specifici o tumore-associati. Perchè la riposta sia efficiente il linfocita deve però anche ricevere un segnale costimolatorio. Le molecole costimolatorie sono generalmente espresse solo su cellule immunitarie, con l'eccezione di B7h, che e' anche espresso su altri tipi cellulari, tra cui cellule endoteliali vascolari e cellule stromali attivate. Ecco perché ICOS ci sembra un buon candidato come molecola costimolatoria potenzialmente espressa da cellule tumorali.

In modelli murini è stato studiato l'effetto dell'espressione ectopica di B7.1 e B7.2 sulle cellule tumorali [Townsend S.E., 1993; Chen L., 1992; Baskar S., 1993]. L'induzione dell'espressione di B7.1 su cellule tumorali mediante trasfezione è sufficiente a indurre rigetto mediato da linfociti T CD8+ e CD4+. Il rigetto inoltre genera memoria immunologica e una conseguente immunità alla successiva induzione dello stesso tipo di tumore. Lo stesso approccio è stato usato per dimostrare che l'espressione ectopica di B7h è in grado di fornire il necessario segnale costimolatorio e di promuovere il rigetto CD8-mediato di fibrosarcoma e plasmacitoma [Wallin JJ, 2001; Liu X, 2001]. Sono già in corso *trials* clinici per testare l'efficacia di vaccini basati su cellule tumorali trasfettate con B7.1 [Antonia SJ, 2002; Raez LE, 2004; Chan L, 2006].

Tutti gli studi finora realizzati hanno usato come modello tumori trapiantabili o indotti chimicamente, mentre mancano dati sul ruolo di ICOS/B7h in modelli di tumore spontaneo con stadi di sviluppo molto più simili al corrispettivo umano, come quello rappresentato dal topo BALB-neuT.

La somministrazione dell'anticorpo anti-ICOS C398.A si topi BALB-neuT si è dimostrato capace di rallentare in modo significativo lo sviluppo del tumore, valutato mediante vari parametri quali il tempo di comparsa dei tumori, la molteplicità tumorale e le dimensioni dei tumori sviluppati. Questi esperimenti non permettono di definire quali meccanismi siano responsabili di questo ritardo. In effetti l'anticorpo C398A lega un epitopo di ICOS in parte diverso rispetto a quello legato dal ligando naturale B7h e si comporta da agonista parziale: *in vitro* infatti ha effetto stimolatorio sulla funzionalità dei linfociti T, ma inferiore rispetto a quello di B7h. L'effetto di C398A *in vivo* potrebbe quindi essere legato all'attività agonista dell'anticorpo su ICOS capace di stimolare funzioni immunitarie aggressive nei confronti del tumore, quali l'induzione di effettori anti-tumorali di tipo TH1, TH17 o CTL.

D’altra parte l’effetto potrebbe invece essere mediato da un’attività antagonista dell’anticorpo sulla interazione tra ICOS e B7h che potrebbe inibire funzioni di ICOS in qualche modo favorevoli allo sviluppo del tumore; ad esempio questa attività antagonista potrebbe ostacolare l’induzione di linfociti Treg capaci di inibire l’immunità anti-tumorale bloccando il signaling di ICOS o il “reverse signaling” di B7h. Indicazioni sul meccanismo di azione dell’anticorpo potrebbero venire dallo studio delle popolazioni linfocitarie anti-tumorali indotte nei topi trattati e non trattati. Una prima analisi dell’assetto delle sottopopolazioni linfocitarie spleniche totali non ha evidenziato nessun effetto degno di nota, per cui sarà necessario approfondire l’analisi valutando funzionalmente le popolazioni linfocitarie dal punto di vista della produzione citochinica, attività citotossica e T regolatoria e indirizzando l’attenzione anche ai linfociti infiltranti il tumore (TIL).

Un approccio alternativo per valutare il meccanismo di azione di ICOS in questo tumore sarà quello di produrre topi HER2/neu+/- difettivi per ICOS (ICOS-/-) incrociando topi BALB-neuT e topi ICOS-/-, che sono già disponibili. Questi esperimenti sono stati iniziati, ma il loro sviluppo è stato rallentato dalla disponibilità di topi ICOS-/- solo nel background genetico C57BL/6, mentre i topi BALB-neuT hanno il background BALB/c. Esperimenti pilota effettuati incrociando maschi BALB-neuT con femmine C57BL/6 ICOS-/- hanno dato risultati incoraggianti, in quanto la prole F1 ha mostrato un trend di ritardo nello sviluppo del tumore il che suggerisce che l’effetto complessivo di ICOS in questo modello sperimentale potrebbe essere quello di favorire lo sviluppo del tumore, piuttosto che contrastarlo. Tuttavia l’elevata eterogeneità genetica di questa prole F1 risulta essere un pesante fattore confondente, per cui si è scelto di ripetere questi esperimenti dopo aver trasferito il genotipi ICOS-/- nel background BALB/c.

Osservazioni conclusive

Gli studi funzionali effettuati hanno permesso di approfondire la conoscenza della coppia recettoriale ICOS/B7h. Tale coppia sembra essere univoca, a differenza di ciò che si osserva per esempio per gli altri membri della famiglia di CD28 per i quali sono stati riconosciuti più ligandi [Burmeister, Y. et al, 2008].

Gli effetti di ICOS e B7h sono modulabili in base alle condizioni del microambiente circostante: a seconda del *pool* citochinico presente o delle altre molecole costimolatorie coinvolte, l'interazione ICOS/B7h può attivare o regolare diversamente la risposta immunitaria. In letteratura prevale l'opinione che ICOS sia coinvolto soprattutto nella modulazione della funzione delle cellule T precedentemente attivate e la maggior parte dei dati disponibili sono stati ottenuti nel modello murino. Nel primo lavoro esposto [Mesturini et al., 2006] abbiamo ottenuto la dimostrazione che nell'uomo ICOS svolge la sua funzione anche in linfociti T CD4+ *naïve*. I risultati ottenuti dimostrano che in queste cellule ICOS potenzia la differenziazione linfocitaria verso funzioni prevalentemente di tipo T_H1, caratterizzate da produzione di IFNγ, quando agisce in sinergia alla stimolazione di CD28 o comunque in presenza di IL-2. Viceversa in assenza di IL-2 la stimolazione di ICOS induce i T *naïve* a differenziare verso funzioni di tipo regolatorio caratterizzate dalla produzione di citochine immunosoppressive come IL-10 e TGFβ.

Il secondo lavoro dimostra che il ruolo di ICOS si estende anche alla differenziazione di linfociti T_H17, agendo a due livelli. Da un lato la stimolazione di ICOS sui linfociti T *naïve* in presenza di IL-1 induce la produzione di IL-17. Dall'altro lato la stimolazione di B7h sulle DC attivate con LPS attiva caspasi 1 e induce la maturazione e secrezione di IL-1β, che è un fattore cruciale per la differenziazione di T_H17. Questa osservazione identifica un ruolo del tutto nuovo di ICOS, ovvero la capacità di modulare l'attività della DC stimolando il *reverse signaling* di B7h.

Nel terzo studio ho voluto analizzare l'azione di ICOS sullo sviluppo di un tumore mammario spontaneo nei topi BALB-neuT interferendo nella interazione ICOS-B7h attraverso la somministrazione dell'anticorpo anti-ICOS C398.4A. Questi esperimenti hanno dimostrato che C398.4 A è in grado di ritardare la cinetica di sviluppo del tumore: tuttavia non permettono di definire quali meccanismi siano responsabili di questo effetto. Infatti l'anticorpo C398.4A lega un epitopo di ICOS in parte diverso rispetto a quello legato dal ligando naturale B7h e si comporta da agonista parziale esercitando un effetto stimolatorio sulla funzionalità dei linfociti T, ma inferiore rispetto a quello di B7h. D'altra

parte il legame di C398.4A ad ICOS interferisce stericamente in modo parziale con l'interazione ICOS.B7h. L'effetto di C398.4A *in vivo* potrebbe quindi essere legato all'attività agonista dell'anticorpo su ICOS capace di stimolare funzioni immunitarie aggressive nei confronti del tumore, quali l'induzione di effettori anti-tumorali di tipo T_H1, T_H17 o CTL. D'altra parte l'effetto potrebbe invece essere mediato da un'attività antagonista dell'anticorpo sulla interazione tra ICOS e B7h che potrebbe inibire funzioni di ICOS in qualche modo favorevoli allo sviluppo del tumore, come ad esempio l'induzione di linfociti Treg.

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Role of NK cells and adaptive immunity in "immunoediting": Recent developments

I. Seren Bernardone

Department of Medical Sciences, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy.

PAPEL DE LAS CÉLULAS NK Y DE LA INMUNIDAD ADAPTATIVA EN LA "INMUNOEDICIÓN": DATOS RECIENTES

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RESUMEN

El papel de los distintos brazos del Sistema Inmune en el control y la eliminación de tumores ha sido objeto de un interés creciente. En este trabajo se discuten, en el marco de la teoría de la "inmunoedición", una serie de datos recientes acerca del papel de la inmunidad en el control de los tumores. En particular, se comentan los datos de Guerra y cols acerca del papel de las células NK en la eliminación de tumores espontáneos en modelos transgénicos de tumorigénesis (Guerra y cols, 2008), y el notable trabajo de Koebel y cols (2007) en el que se muestra el papel de la inmunidad adaptativa en el mantenimiento como una enfermedad crónica de los tumores inducidos por agentes químicos.

PALABRAS CLAVE: Vigilancia inmunológica/ Inmunoedición/ Inmunidad Tumoral/ Células NK/ Linfocitos T.

ABSTRACT

The role of the different arms of the Immune System in the control and elimination of tumors has been the subject of increasing interest in recent years. Here, some recent findings adding support to the role of NK cells in the elimination of spontaneous tumors in transgenic models of tumorigenesis (Guerra et al, 2008), or the role of adaptive immunity in the maintenance of chemically induced tumors as a chronic disease (Koebel et al, 2007) will be discussed within the frame of the "immunoediting" theory.

KEY WORDS: Immune surveillance/ Immunoediting/ Tumor immunity/ NK cells/ T lymphocytes.

INTRODUCTION

Cancer is a disease initiated by a series of cumulative genetic and epigenetic changes that occur in a normal cell. However, in addition to the malignant cell itself, cancer is a disease of microenvironment and immunity. Multiple signals delivered within the tumor microenvironment by stromal and endothelial cells, and immune cells are critical factors in determining the progression versus dormancy or destruction of an initiated lesion and also whether metastasis may occur[1].

Here, some recent findings on the role of the immune system in elimination and/or contention of tumor growth will be reviewed. Particularly, recent data concerning the role of NK cells in the elimination of spontaneous tumors[2,3], or the role of adaptive immunity in the maintenance of tumors as a chronic disease[4] will be discussed.

The concept that the Immune System protects the host against cancer was first proposed by Erlich in 1909 and modified in the 1950s by Burnet and Thomas[5] The immunosurveillance theory of Burnet and Thomas proposed that adaptive lymphocytes could respond to and reduce tumor growth by recognizing tumor antigens. However, the concept fell out of favor when studies in the 1980s indicated that tumors failed to develop more rapidly in nude mice (which lack T cells and B cells, but not NK cells) than in wild-type mice. It was resurrected in the 1990s, when a body of evidence emerged indicating that immunodeficient mice were at greater risk for spontaneus tumor development[5].

Four classes of cells have been established to have key roles in the immune response against tumors, and consequently the Immune System is totally involved in this action. These cells are: Natural Killer (NK) cells, that provide innate immune response; CD8+ T lymphocytes, that represent the adaptive immune response; NKT cells, that connect the two classical type of immune response and so are usually regarded as "transitional" immune-responses; Tregs, that recognise an antitumor immune response as an autoimmunity response and manage to inhibit it (Figure 1).

THE ROLE OF NK CELLS

Since their discovery, a large number of studies have demonstrated natural killer (NK) cell-mediated lysis of different types of tumor cells *in vitro* and *in vivo*, but, for a long time, it was unknown how NK cells recognized tumor cells, as well as other aberrant cells. Over the last 15 years, however, a large number of germline-encoded NK cell-activation and –inhibitory receptors have been discovered. Among the activating receptors, natural killer group 2 member D (NKG2D) is one of the best characterized: It is a type II transmembrane-anchored glycoprotein expressed as a disulfide-linked homodimer on the surface of almost all NK cells as well as some $CD8^+$ $\alpha\beta^+$ T cells, $\gamma\delta^+$ T cells, NKT cells, and a small subset of $CD4^+$ $\alpha\beta^+$ T cells. NKG2D ligands are frequently expressed on primary tumor cells, tumor cell lines and some cells infected by pathogens, which become sensitive to NK killing. A DNA-damage pathway regulates NKG2D ligand expression. Indeed, ligand-transfected tumor cells have been shown to be rejected *in vivo* in an NKG2D-dependent fashion. Therefore, immunological recognition of developing tumors in the host may result not only from the recognition of specific antigens presented by MHC class I and class II molecules, but also by cellular-stress-induced ligands expressed on transformed cells but not on normal cells. Guerra and colleagues state that NKG2D deficiency promotes the development of spontaneous tumors[2]. Notably, however, genetic NKG2D deficiency does not seem to affect the incidence of carcinogen-induced sarcomas[2]. Whereas these findings are consistent with a role of NKG2D in tumor surveillance, they also show that some tumors may evade it, for example by shedding high amounts of soluble NKG2D ligands, which are believed to cause downregulation of NKG2D on the surface of lymphocytes. In the future it is not unlikely that more cancers will be treated with drugs or other immunomodulatory agents that affect the function of NK cells (and other immune cells), either directly or indirectly. Furthermore, certain forms of cancer may be subject to treatment with adoptively transferred NK cells, and cancer therapy may also benefit from novel strategies aimed at inducing the expression of ligands for activating immune receptors in tumors[2,3].

THE ROLE OF NKT CELLS

NKT cells have begun to yield increasingly interesting results concerning tumor immunity. NKT cells and $\gamma\delta^{++}$ T cells may "sit" between innate and adaptive immunity. Like adaptive cells, they bear receptors encoded by somatically rearranged genes, yet like innate cells, they generally lack distinct potential for establishing antigen-specific clonal memory. The type of response mounted by such cells has been referred to as "transitional immunity", and its importance in tumor surveillance is evident in the antitumor activity of the NKT cell agonist α-galactosylceramide, or the observation that mice deficient in γδ T cells are much more susceptible to several protocols of skin carcinogenesis induction[6].

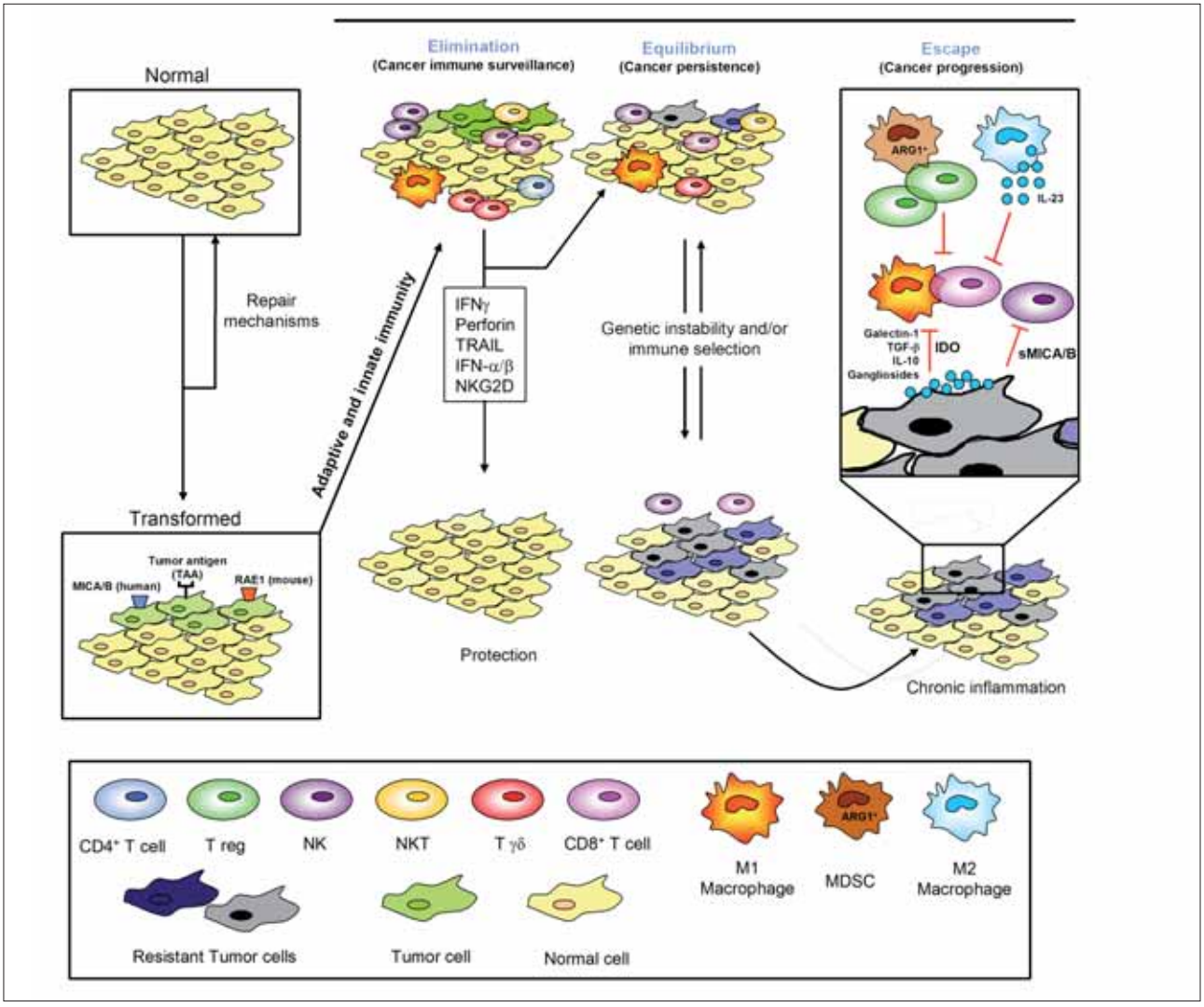

Figure 1. *How tumor escape overcomes the control and elimination mechanisms of innate and adaptive immunity.*

THE ROLE OF CD8+ T CELLS

CD8+ T cells are key to our ability to control intracellular infections and cancer. They act as cytolytic T lymphocytes (CTLs) that not only eliminate in a cytotoxic manner the infected/tumor cells but also carry out regulatory functions, being capable of either suppressing or supporting immune responses. Whereas the mechanism of the suppressive activity of CD8+ T cells is far from clear, it has been shown that perforin- and granzyme-dependent elimination of antigen-carrying dendritic cells (DCs) by antigen-specific CD8+ T cells can act as a suppressive mechanism, providing self-limiting character to CTL responses and restricting efficacy of vaccinaton. On the other hand, CD8+ T cells can also activate DCs, this "helper" function depending on their ability to produce IFN-γ and to promote production of IL-12p70 by DCs[7].

TUMOR ESCAPE: TREG AND MYELOID-DERIVED SUPPRESSOR CELLS (MDSC)

T cell tolerance plays an important role in tumor escape and is one major obstacle, limiting the effectiveness of cancer vaccines. Tumors express antigens that should induce immune-mediated rejection, yet spontaneous rejection of established tumors is rare because tumors can actively fight and defeat host immunity. The active mechanisms involved in the suppression of host immunity include altering APC function, fostering dysfunctional T cell co-signaling,

or generating an immune-subversive cytokine milieu. Given these premises, reducing tumor-driven immune suppression should be clinically beneficial.

The current paradigm around which most cancer immunotherapies have been developed arose from observations made in infectious diseases. So, many of these vaccines have been developed against tumor associated antigens (TAA) shown to elicit antigen-specific antitumor $CD8^+$ CTLs. Unfortunately, although immunity against tumors and pathogens share some characteristics, they also have substantial differences. According to the current paradigm, tumor express TAA that can be captured by professional APCs, notably DCs, which then prime naïve T cells (through the expression of co-signaling molecules, the production of soluble factors, or other mechanisms) to become antigen-specific $CD8^+$ CTLs. These cells, when found in appropriate numbers and directed against the appropriate antigens, are then able to eradicate the tumor. According to this paradigm, simply supplying more of the missing elements should result in immune rejection of the tumor. Numerous anticancer immunotherapeutic strategies have been developed based upon these premises, including infusing additional tumor antigen or antigen-pulsed APCs, supplying T cells generated from tumor-infiltrating lymphocytes, T cells together with a soluble growth factor, T cells activated ex vivo with cytokines, or T cells engineered to express receptors for specific TAA and boosting the effect of co-signaling molecules or activating cytokines. The fundamental difference between infection and cancer is that the first has an extrinsic origin while the second is intrinsic (self) to the individual. Because tumors are intrinsic, generating effective antitumor immune responses requires mounting a substantial autoimmune attack, which involves breaking self-tolerance. Indeed, for example, tumors actively fight back by producing immunosuppressive factors such as IL-10, TGF-β and VEGF.

APCs are primary responsible for the induction of tumor-induced T-cell tolerance. A group of $Gr\text{-}1^+CD11b^+$ MDSCs have been identified as primarily responsible for tumor-associated $CD8^+$ T-cell tolerance. These MDSC are immature cells, comprising precursors of macrophages, granulocytes, dendritic cells (DCs) and myeloid cells at earlier stages of differentiation, and they can suppress immune response *in vitro* through direct cell-cell contact, and antigen-specific MHC class I-restricted tolerance of $CD8^+$ T cells *in vivo*. Accumulation of these cells has been described in individuals with cancer, and this pathway may explain the difficulties in maintaining the antigen-specific immune response after vaccination in cancer patients. There are two basic mechanisms to induce T-cell tolerance, namely deletion and anergy, yet MDSCs seem to act through another, largely unknown mechanism. Nagaraj and colleagues, using an experimental *in vivo* model, had shown that MDSCs, by generating reactive oxygen species (ROS) and peroxynitrite, induce modification of TCR and CD8 molecules, resulting in the loss of ability of $CD8^+$ T cells to bind pMHC. MDSCs do not affect the expression of TCR or CD8 molecules on the surface of T cells. This third mechanism may be acting in many pathological conditions in addition to cancer, including infection, inflammation and trauma, all of them associated with the accumulation of MDSCs overproducing peroxynitrite[8].

$CD4^+CD25^+$ regulatory T lymphocytes (Treg) are the second important actors of tumor-driven immune evasion providing prototypical targets to test new anticancer treatment strategies. Although suppressor T cells were discovered about 40 years ago, it was Sakaguchi the first to describe phenotypical characteristics to identify these cells, in 1995. He demonstrated the active suppressive potential of a population of $CD4^+$ cells expressing high levels of CD25, now called Tregs. Tregs suppress autoreactive T cells by a not yet clearly understood contact-dependent mechanism.

Interestingly, suppression of tumor immunity by Tregs was first described in the early 1980s but was largely ignored. However, the recent demonstration that depletion of Tregs in mouse improves endogenous immune-mediated tumor rejection and tumor antigen-specific immunity has boosted the interest in the role of Tregs in tumor immunity. It was soon demonstrated that Treg depletion augments tumor immunotherapy, including vaccination and CTLA4 blockade. It is currently thought that Tregs suppress $CD4^+$ and $CD8^+$ T cell responses by cell-cell contact mechanisms and/or by the production of immunosuppressive soluble factors like IL-10 or TGF-β. It has been shown that the number of Tregs is increased in the blood of patients with different kinds of tumors, and these cells populate the tumor mass and the draining lymph nodes. Furthermore, in some cases there seems to be an inverse correlation between the presence of intra-tumor Treg and patient survival, and Treg depletion has been useful to improve the results of anti-cancer immunotherapy. The increased number of Tregs might be due to either tumor immunity being considered as an autoreactive response, or as a response to an inflammatory condition, or to other as yet undetermined factors. To be therapeutically useful, it is important to study these cells in the appropriate anatomical compartment, and to deplete them locally, i.e., within the tumor or the draining lymph nodes. In this regard, it is important to note that there are two phenotypically identical populations of $CD4^+CD25^+$ Tregs, i.e. adaptive and natural. Natural Treg arise in the thymus as a safeguard against autoimmunity, while adaptive arise during inflammatory processes including infections

or cancer. Therefore, Tregs that infiltrate the tumor microenvironment are probably adaptive and they are induced to differentiate *in situ* into several subpopulations by tumor cells or by other cells, such as intratumor DCs. It should be also mentioned that Tregs can also inhibit the function of NK and B cells[9].

THE THEORY OF THE "IMMUNOEDITING"

The complex interactions between different cells of the Immune System and between these cells and the microenvironment in the course of antitumor responses are well described by the term "immunoediting" (Figure 1). Stromal cells in the solid tumor microenvironment nourish and often outnumber the tumor cells themselves: Endothelial cells, fibroblasts, inflammatory cells and T regulatory immune cells are all generally abundant. Immunoediting starts with recognition and destruction of transformed cells that have acquired genetic damage (immunosurveillance). The 3 stages of immunoediting lead to control, stasis or outgrowth of a tumor and are usually named as follows: **1. Elimination:** The immune system recognizes tumor cells and destroys them. **2. Equilibrium:** The immune system is unable to completely destroy the tumor but converts it in a quiet mass of unproliferating cells. **3. Escape:** The tumor evades the effector mechanisms of the Immune System and proliferates without control (Figure 1)[1,5].

So, one interesting assert of this theory is that, in addition to the Immune System's capacity to destroy and shape cancer, immunity can also have cancer under control for long periods of time by a process called *equilibrium.* Equilibrium is a component of cancer *immunoediting* because cells in equilibrium are highly immunogenic, whereas those cells exiting equilibrium and becoming growing tumors have attenuated immunogenicity. These results then place this process temporally between elimination and escape. Whereas elimination requires elements from both the innate and the adaptive immune response, *equilibrium is solely maintained by adaptive immunity*. Immune mechanisms can influence cancer growth both quantitatively and qualitatively, the quality and quantity of the immune reaction being reliable prognostic indicators of cancer patient survival[10]. Extensive experimental support exists nowadays about the elimination and escape processes because immunodeficient mice develop more carcinogen-induced and spontaneous cancers than wild-type mice, and tumor cells from immunodeficient mice are more immunogenic than those from immunocompetent mice. In contrast, the equilibrium process has been inferred largely from clinical observations, including reports of transplantation of undetected (occult) cancers from organ donor into immuno-suppressed recipients.

THE ROLE OF ADAPTIVE IMMUNITY IN IMMUNOEDITING

In December 2007, Koebel and colleagues published interesting results that extend our knowledge of the role of the Immune System in the equilibrium state, providing experimental support to this process and opening the possibility that tumors could be treated like a chronic disease[4]. They demonstrated that the equilibrium estate is mechanistically distinguishable from elimination and escape and that neoplastic cells in equilibrium are transformed but proliferate poorly *in vivo*. The aim of these authors was also to find molecular markers to characterize the equilibrium state and the other two phases of the immunoediting process. Indeed, in recent years, immunotherapeutic approaches to treat cancer, with either adoptive transfer of immunity or stimulation of the endogenous immune system, have shown increasing promise. Clearly, the goal of cancer therapy is to kill residual tumors that cannot be excised surgically. Being of host origin, cancer cells share features of the host that make effective treatments difficult, due to side effects that limit the therapeutic window. Moreover, the plastic nature of tumors make them remarkably resilient in rebounding from clinical radiotherapy and chemotherapy treatments that are traditionally used. The tumor cells develop resistance under the selective pressures posed by cytotoxic agents. Genetic plasticity is a key characteristic of cancer cells, so that successful targeting requires the application of multiple agents that target different survival mechanisms[11]. A solution may be to redirect the focus of the attack from the tumor cells to the environment that sustains their growth and survival (i.e., stromal cells in the tumor environment are not genetically plastic), or to engage the immune system.

It is in this point where the importance of the work of Koeble and colleagues resides: Even treatments that did not cure cancer but rather converted it to a long-term subclinical or at least manageable condition, would represent a resounding success. The key question is how can a tumor outrun an activated Immune System, so that the balance might be tipped back in favor of the Immune System. Some cancer immunologists are using the term "immune checkpoint" to refer to the negative-acting suppression pathways that prevent activation of the immune system.

The strategy of Koebel and colleagues was to use C57BL/6 and 129/SvEv mouse colonies established in two different laboratories to inject age- and sex-matched groups with 3'-methylcholanthrene (MCA) to induce sarcoma formation in the mice (primary tumorigenesis model). The interest of the authors then focused on the mice that did not develop progressively growing tumors, so that they eliminated all mice that had expanding tumors by 200 days. The animals

that had only small stable masses at the injection site (supposed to be in an equilibrium state) were then treated with either control immunoglobulins or a mixture of monoclonal antibodies to deplete $CD4^+$ and $CD8^+$ T cells and to neutralize IFNγ or IL-12p70. They found that sixty per cent of these mice developed progressively growing tumors, whereas no growth was observed in control mice. Thus, the treatment facilitated the expansion of pre-formed occult cancer cells. Importantly, suppression of NK cell function did not induce tumor outgrowth. This finding indicated that equilibrium involves only adaptive immunity mechanisms. To examine this process more carefully, the authors then used mice deficient in recombinant-activating genes ($Rag1^{-/-}$ or $Rag2^{-/-}$ mice) which have an innate, but not an adaptive immune response. The results showed that sarcoma formation in $Rag^{-/-}$ mice was essentially complete within 200 days of MCA exposure. In addition, the mean time to tumor formation in MCA-treated $Rag^{-/-}$ mice differed strickingly from that found in MCA-treated wild-type mice, rendered immunodeficient at day 200 using the anti-CD4/-CD8/-IFNγ mixture (105±5 days versus 25±6 days). Taken together, these data argue strongly against continuous *de novo* transformation as the mechanism underlying the late tumor outgrowth in wild-type mice after immunodepletion. Rather, they raise the possibility that at least some of the proposed tumor-promoting actions of chronic inflammation may be a result of interfering with adaptive immunity's capacity to hold unnoticed cancers in equilibrium. Furthermore, the immunohistochemical staining of the tumor stable masses in MCA-treated wild-type mice detected a population of atypical fibroblast-like cells that formed tumors when injected into $Rag2^{-/-}$ mice. This fact demonstrates the existence of occult tumor cells and explains how an occult cancer can be transplanted from a donor organ to a recipient[12], because tumor cells held in equilibrium in the donor may grow in a recipient that is at the same time naïve to the antigens of the transplanted tumor cells and immunosuppressed.

DISCLOSURES

The author declare no financial conflicts of interest.

CORRESPONDENCE TO:
Ilaria Seren Bernardone
Department of Medical Sciences
Amedeo Avogadro University of Eastern Piedmont
Via Solaroli 17
I-28100 Novara, Italy
Phone: +39-0321-660644
E-mail: ilaria.seren@med.unipmn.it

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ORIGINAL ARTICLE

Variations of the perforin gene in patients with multiple sclerosis

G Cappellano[1,11], E Orilieri[1,11], C Comi[2,3], A Chiocchetti[1], S Bocca[1], E Boggio[1], IS Bernardone[1], A Cometa[4], R Clementi[1], N Barizzone[1], S D'Alfonso[1], L Corrado[1], D Galimberti[5], E Scarpini[5], FR Guerini[6], D Caputo[7], D Paolicelli[8], M Trojano[8], L Figà-Talamanca[9], M Salvetti[9], F Perla[10], M Leone[1,2], F Monaco[1,2] and U Dianzani[1]

[1]*Department of Medical Sciences, Interdisciplinary Research Center of Autoimmune Diseases, A Avogadro University of Eastern Piedmont, Novara, Italy;* [2]*Department of Neurology, A Avogadro University of Eastern Piedmont, Novara, Italy;* [3]*Neurorehabilitation Center, ML Novarese, Moncrivello, Italy;* [4]*Laboratory of Transplant Immunology, Division of Pediatric Hematology/Oncology, IRCCS Policlinico San Matteo, Pavia, Italy;* [5]*Department of Neurological Sciences, University of Milan, IRCCS Fondazione Ospedale Maggiore Policlinico, Milan, Italy;* [6]*Laboratory of Molecular Medicine and Biotechnology, Don C Gnocchi Foundation ONLUS, IRCCS, Milan, Italy;* [7]*Multiple Sclerosis Unit, Don C Gnocchi Foundation ONLUS, IRCCS, Milan, Italy;* [8]*Department of Neurological and Psychiatric Sciences, University of Bari, Bari, Italy;* [9]*Neurological Center of Experimental Therapy, University of Rome, La Sapienza, S Andrea Hospital, Rome, Italy and* [10]*Neurology Division, S Croce e Carle Hospital, Cuneo, Italy*

Perforin is involved in cell-mediated cytotoxicity and mutations of its gene (PRF1) cause familial hemophagocytic lymphohistiocytosis (FLH2). PRF1 sequencing in 190 patients with multiple sclerosis and 268 controls detected two FLH2-associated variations (A91V, N252S) in both groups and six novel mutations (C999T, G1065A, G1428A, A1620G, G719A, C1069T) in patients. All together, carriers of these variations were more frequent in patients than in controls (phenotype frequency: 17 vs 9%, P = 0.0166; odds ratio (OR) = 2.06, 95% confidence interval (CI): 1.13–3.77). Although A91V was the most frequent variation and displayed a trend of association with multiple sclerosis (MS) in the first population of patients and controls (frequency of the 91V allele: 0.076 vs 0.043, P = 0.044), we used it as a marker to confirm PRF1 involvement in MS and assessed its frequency in a second population of 966 patients and 1520 controls. Frequency of the 91V allele was significantly higher in patients than in controls also in the second population (0.075 vs 0.058%, P = 0.019). In the combined cohorts of 1156 patients and 1788 controls, presence of the 91V allele in single or double dose conferred an OR = 1.38 (95% CI = 1.10–1.74). These data suggest that A91V and possibly other perforin variations indicate susceptibility to MS.
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Keywords: *MS; perforin; autoimmune diseases*

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system.[1] Its clinical course varies; at onset, approximately 15% of patients display a primary progressive (PP) form, whereas the remainder start out with a relapsing remitting (RR) form and most of them switch to a secondary progressive (SP) form within 10–30 years.[2] Both environmental and genetic factors are involved in the development/progression of MS and several studies point to a complex inheritance involving interactions between combinations of loci that may influence the immune response.[3,4]

Demyelination is obviously a pathological hallmark of MS, but recent evidence has suggested that the clinically relevant cause of functional disability is injury to the axon.[5] This neurodegenerative model posits that demyelination is a permissive factor that creates an environment in which the axon becomes susceptible to injury mediated either by loss of axo–glial trophic interactions or immune-mediated attack of the denuded axon. The cellular effectors responsible for injuring demyelinated axons are currently unidentified. The fact that $CD8^+$ T cells are the most abundant lymphocytes within MS lesions[6] and correlate with axon injury[7] suggests that class I-restricted cytotoxic T cells (CTL) may be the culprit.

Cytolytic granules of $CD8^+$ CTL and natural killer (NK) cells contain perforin and granzymes, and are released on the target cell upon its recognition by the cytotoxic cell. Perforin polymerizes on the target cell membrane and forms pores allowing entry of granzymes

Correspondence: Professor U Dianzani, Department of Medical Sciences, Interdisciplinary Research Center of Autoimmune Diseases, A Avogadro University of Eastern Piedmont, Via Solaroli 17, Novara I-28100, Italy.
E-mail: dianzani@med.unipmn.it
[11]These authors contributed equally to the work.
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that trigger apoptosis of the target cell by cleaving caspases.[8]

Biallelic loss-of-function mutations of the perforin gene (*PRF1*) have been classically associated with about 30% of cases of familial hemophagocytic lymphohistiocytosis (FLH2), a rare life-threatening immune deficiency that occurs in infants and young adults.[9,10] FLH2 has been classically ascribed to decreased capacity of CTL and NK cells to clear viral infections; viral persistence is thought to cause the lymphoproliferative pattern. FLH2 is a recessive disease and subjects carrying heterozygous *PRF1* mutations are generally healthy. However, some heterozygous variations may favor development of autoimmune diseases. This has been initially suggested for the autoimmune lymphoproliferative syndrome (ALPS), a rare pediatric autoimmune disease due to defective function of the Fas death receptor involved in both downmodulation of the immune response and cell-mediated cytotoxicity.[11] ALPS is primarily due to mutations of the Fas gene or other genes involved in Fas function, but other genetic factors may concur. We have detected two FLH2-associated amino-acid substitutions of *PRF1* that are associated with ALPS, that is, N252S and A91V.[11] A subsequent work on patients with type 1 diabetes mellitus (T1DM) detected association with N252S, but not A91V, and a patient displayed a novel mutation causing a P477A amino-acid change decreasing NK function.[12] This work was aimed to evaluate whether *PRF1* also contributes to MS development in the light of recent studies showing that a region of chromosome 10q22.1, located near *PRF1*, may be a susceptibility locus for MS.[13–15]

Results

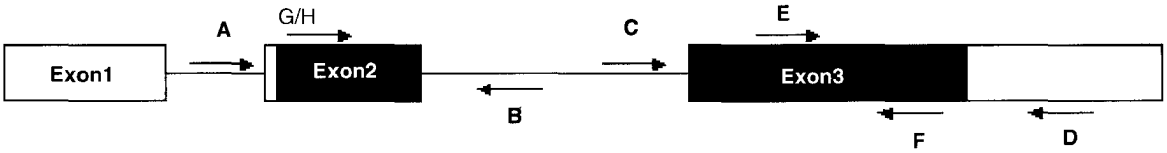
Analysis of the whole coding region of PRF1

The entire coding region of *PRF1* was sequenced in 190 MS and 268 controls to look for variations associated with FLH2 or novel variations (Figure 1). Four missense variations were detected, that is, C272T (rs35947132), A755G (rs28933375), G719A and C1069T (numerations are referred to the GenBank cDNA clone M28393, ATG = +1) causing A91V, N252S, R240H and R357W amino-acid substitutions, respectively. A91V and N252S are variations previously associated with FLH2, whereas R240H and R357W are new.

Four other novel variations, C999T, G1065A, G1428A and A1620G, were detected, but they were synonymous variations (P333P, P355P, G476G and Q540Q, respectively); analysis of their putative effect on splice sites using the Spliceview software and ESEfinder scoring matrix showed that only A1620G (Q540Q) may have an effect by creating a novel acceptor splice site (Spliceview) and a novel binding site for Ser/Arg-rich proteins (ESEfinder), a family of conserved splicing factors.

Finally, we detected the two nucleotide variations, C822T (rs885821) and T900C (rs885822), previously reported as common polymorphisms not associated with FLH2; they did not change the amino acid, nor influence the splicing sites. Their frequency was similar in the patients and the controls. Two other synonymous variations (G435A and A462G) are known to be in perfect linkage disequilibrium with N252S and were in fact only detected in the two subjects (one patient and one control) carrying this variation.[11]

A91V was detected 29 times in 26 patients (23 heterozygotes, 3 homozygotes) and 23 controls (heterozygotes); N252S in 1 patient and 1 control (heterozygotes); R240H in 2 patients (heterozygotes); R357W, P333P, P355P, and G476G in 1 patient each (heterozygotes); and Q540Q in 1 patient (homozygote). The R357W and P355P carriers were also heterozygous for A91V, and the two variations were found to be on different alleles by allele-specific PCR. All together, frequency of the FLH2-associated and novel variations was higher in patients than in controls (allele frequencies: 0.100 vs 0.045, $P = 0.0016$; phenotype frequency: 17 vs 9%, $P = 0.0166$; odds ratio (OR) = 2.06, 95% confidence interval (CI): 1.13–3.77; Table 1).



Nucleotide variation	Predicted amino acid change	Exon	Previous reports
C272T	A91V	2	FLH2-associated
A755G	N252S	3	FLH2-associated
G719A	R240H	3	Novel
C999T	P333P	3	Novel
G1065A	P355P	3	Novel
C1069T	R357W	3	Novel
G1428A	G476G	3	Novel
A1620G	Q540Q	3	Novel

A	5' -cccttccatgtgccctgataatc-3'
B	5' -ccaatcaaacttggaggctgct-3'
C	5' -ccagtcctagttctgcccacttac-3'
D	5' -gtatgcttggactgaaggggttc-3'
E	5' -caggtcaacataggcatccacg-3'
F	5' -gaacagcaggtcgttaatggagg-3'
G	5' -ctccagcgcctgcctccggC-3'
H	5' -ctccagcgcctgcctccggT-3'

Figure 1 Graphical representation (not in scale) of the *PRF1* gene, primers used for typing and the variations found in MS patients. The upper panel shows a scheme of the gene and the relative position of the primers; boxes represent the exons (the coding region is shown in black), lines the introns. Letters and arrows indicate the primers used to amplify and sequence the gene (see Materials and methods) and their sequence is shown in the lower right table. The lower left panel shows a summary of the FLH2-associatcd (*) and novel *PRF1* variations detected in 190 MS patients and 268 controls.

Table 1 Summary of the genotypes of 190 MS patients and 268 controls carrying *PRF1* variations

Allele 1	*Allele 2*	*MS (n = 190)*[a]	*Controls (n = 268)*[a]
A91V	A91V	3	0
A91V	R357W	1	0
A91V	P355P	1	0
Q540Q	Q540Q	1	0
A91V	wt	21	23
N252S	wt	1	1
R240H	wt	2	0
P333P	wt	1	0
G476G	wt	1	0
Total		32 (17%)	24 (9%)
		[b]OR = 2.06, 95% CI: 1.13–3.77; $P = 0.0166$	

Abbreviations: CI, confidence intervals; MS, multiple sclerosis; OR, odds ratio; wt, wild type.
[a]Number of subjects (frequency in the brackets).
[b]OR and 95% CI limits; *P*-values are two-tailed.

The PolyPhen algorithm was used to predict the functional effect of the two novel R240H and R357W missense variations and showed that both may damage the function and structure of the protein (R240H: score = 2.335; R357W: score = 2.690). Therefore, we directly assessed whether R240H affects perforin function by evaluating NK activity in the 2 patients carrying the variation and 15 controls. Results showed that, at low effector/target ratios, NK activity was defective in one patient and in the low level (that is, within the first quartile) of the normal range in the other (Figure 2). This analysis was not performed in the R357W carrier because his cells were not available. Intriguingly, both patients carrying R240H displayed an early switch from the RR to the SP course (5 and 6 years from onset, respectively) and a multiple sclerosis severity score (MSSS) of 7.65 and 7.38, respectively. By contrast, this aggressive clinical evolution was not displayed by the R357W carrier.

Search for the A91V variation in a second population of patients and controls

Although A91V was the most frequent variation and displayed a trend of association with MS in the first population of patients and controls (frequency of the 91V allele: 0.076 vs 0.043, $P = 0.044$), we used it as a marker to confirm *PRF1* involvement in MS, and assessed its frequency in a second independent population of 966 patients and 1520 ethnically and geographically matched controls. The 91V allele was carried by 138 patients (131 heterozygotes and 7 homozygotes) and 168 controls (160 heterozygotes and 8 homozygotes) and its frequency was significantly higher in patients than in controls (0.075 vs 0.058%, $P = 0.019$). In the combined cohorts of 1156 patients and 1788 controls, presence of the 91V allele in single or double dose conferred an OR = 1.38 (95% CI = 1.10–1.74; Table 2).

No differences were found between subjects carrying or not carrying A91V in terms of gender distribution, MS clinical form (RR, PP and SP) and MSSS (data not shown). Moreover, frequency of the MS susceptibility allele HLA-DR15 was not different in patients carrying A91V or not, as HLA-DR15 was carried by 32% of patients with the 91V allele, 29% of patients without it and 12% of the controls.

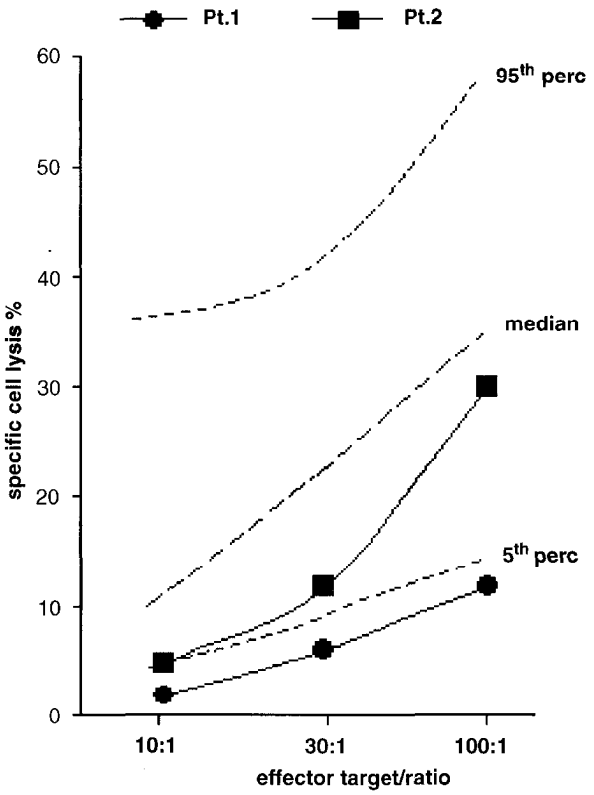


Figure 2 NK activity in PBMC of MS patients carrying the R240H perforin variations and controls. NK activity was assessed at the 100:1, 30:1, and 10:1 effector/target (E:T) ratios; continuous lines indicate patients; stripped and dotted lines indicate the median values and interquartile ranges of 15 controls.

Table 2 Genotype frequencies of A91V in the combined cohorts of MS patients ($n = 1156$) and healthy controls ($n = 1788$)

Genotypes	*MS (n = 1156)*[a]	*Controls (n = 1788)*[a]
AA	992 (0.858)	1597 (0.893)
AV	154 (0.133)	183 (0.102)
VV	10 (0.009)	8 (0.005)
AV+VV vs AA	[b]OR = 1.38, 95% CI = 1.10–1.74; $P = 0.005$	

Abbreviations: CI, confidence intervals; MS, multiple sclerosis; OR, odds ratio.
[a]Number of subjects; frequencies are shown in the brackets. Genotypic distribution did not deviate significantly from the Hardy–Weinberg equilibrium in any group (data not shown).
[b]OR and 95% CI limits; *P*-values are two-tailed.

Discussion

Multiple sclerosis is a complex disease that is probably the result of multiple genetic and environmental factors. Several genes have been involved in its development,[4] and some of them are important in the immune response. This work shows that *PRF1* may also be involved, as MS patients displayed higher frequency of *PRF1* variations

than the controls. This confirms data obtained by other authors showing that the chromosome region 10q22.1, where *PRF1* is located, contains susceptibility genes for MS development.[13–15]

The most frequent variation was A91V, as frequency of the 91V allele was increased in two independent populations of MS patients than in the respective controls, and increased the risk of MS by about 1.4-fold in the combined cohorts. By contrast, A91V did not seem to influence the disease course as MSSS was not different between patients with or without A91V. Studies based on analysis of cytotoxic lymphocytes from A91V carriers or rat basophil leukemia cells transfected with variants of the perforin cDNA have shown that A91V decreases perforin function by altering its conformation, decreasing its cleavage to the active form and increasing its degradation.[16,17] Risma *et al.*[18] classified A91V as a class 1 missense mutation with limited functional impact that allows partial maturation of the protein. Voskoboinik *et al.*[19] have recently used a complementation assay with perforin-knockout primary CTL to show that A91V reduces both the steady-state level of perforin expression in effector cells ('presynaptic' dysfunction) and its intrinsic lytic capacity on target cells, and also displays some dominant-negative effect on the wild-type protein ('postsynaptic' dysfunction).

Our previous work showed that frequency of the 91V allele was also increased in an incomplete variant of ALPS, whereas N252S was associated with the typical form of ALPS and T1DM.[11,12] The functional significance of N252S is debated, but we showed that it may be associated with decreased NK activity in the early childhood.[11,12] Although frequency of N252S was apparently not different in MS and controls, it is possible that *PRF1* variations favor development of several autoimmune diseases, with differences reflecting their effects on perforin function. A91V has also been associated with other immune diseases, such as lymphomas and acute childhood lymphocytic leukemia, and atypical (late-onset) FLH2.[20,21]

Besides A91V and N252S, we detected two new missense *PRF1* mutations in MS patients that cause R240H and R357W amino-acid substitutions. R240H occurs nearby N252S within the membrane-attack complex, a region critically involved in the pore-forming activity of perforin.[22] However, analysis of NK activity in the two R240H carriers showed that it was near the low limit of the normal range. Although both carriers were heterozygous, we suggest that R240H causes a mild decrease of perforin function without exerting a dominant-negative effect on the wild-type form. R357W is located in the same domain, but we could not evaluate its functional effect because fresh cells from the carrier were not available. However, both R357W and R240H were predicted to damage perforin function and structure by *in silico* analysis with the PoliPhen program.

Four other novel mutations were detected in MS patients, but they were synonymous (P333P, P355P, G476G, Q540Q). Q540Q may have an effect on RNA splicing, as it seems to create a new acceptor splice site. The others did not influence canonical splicing sites, but they might theoretically influence perforin expression by disturbing exonic splicing enhancers, mRNA processing and transport, efficiency of codon usage by tRNA stability of mRNA secondary structure, protein folding or interaction with microRNA.[23–28] An alternative possibility is that they do not have a direct effect, but they are linkage disequilibrium with other unknown *PRF1* mutations in the 5′ UTR. However, we could not assess perforin expression because fresh cells from the carriers were not available.

Besides A91V, the other mutations are too rare to draw conclusions about their individual association with MS, but they raise the possibility that the overall effect of *PRF1* variations on MS development may be substantially higher than that detected by A91V alone. In line with this possibility, these variations conferred a global OR = 2.06 for MS development in the first population of patients and controls whose entire *PRF1*-coding region was sequenced. It is intriguing that two patients were compound heterozygous for A91V, and R357W or P355P, respectively, and another patient was homozygous for Q540Q, which raises the possibility that the biallelic variations may have contributed to their MS. The MS association with several rare *PRF1* variations is in line with reports on systemic lupus erythematosus and inflammatory bowel disease indicating that private/rare variations as well as common polymorphisms of other genes may be important in common complex diseases.[29,30]

Perforin-mediated cytotoxicity has been classically associated with clearance of virus-infected cells. Therefore, it is possible that defects of perforin activity favor MS development by delaying virus clearance, which may favor development of crossreactions between viral and self-antigens by molecular mimicry. In this context, it is noteworthy that EBV infections are crucial in FLH2 pathogenesis, and have also been suggested to be important as triggering factors in MS.[31,32]

On the other hand, an increasing bulk of data suggests that perforin and cell-mediated cytotoxicity may also be involved in downmodulation of the immune response. This regulatory activity may involve several mechanisms including perforin-mediated killing of effector lymphocytes and antigen-presenting cells. Defective immune response switching off may favor both lymphocyte accumulation and autoimmunity.[8,33–36] It is noteworthy that involvement of inherited defects of the immune response switching off in MS development may not be limited to *PRF1*, but may also involve defective apoptosis of activated lymphocytes induced through the Fas or the activation-induced cells death (AICD) mechanisms. This possibility is suggested by our previous work showing that substantial proportions of MS patients carry inherited defects of Fas function similar to those displayed by ALPS patients.[37] Moreover, several reports detected high serum levels of osteopontin, a cytokine capable to inhibit AICD, in MS patients and we found that this is partly associated with variants of the osteopontin gene.[38–41]

In conclusion, this work suggests that *PRF1* variations may be a predisposing factor for MS by affecting either the antiviral response or the immune response switching off. Defects of both of these functions may favor development of autoimmunity by prolonging the immune response and increasing the risk of crossreactions between viral and self-antigens. Similar defects may be caused also by alterations of other genes and may be a general predisposing factor for autoimmunity.

Materials and methods

Patients

We analyzed two independent cohorts of Italian patients (391 men, 765 women; M/F: 1/1.96) with MS, diagnosed according to McDonald *et al.*'s criteria[42] and randomly selected ethnically matched healthy controls. The first population was composed of 190 patients and 268 controls, the second by 966 patients and 1520 controls.

Patients were consecutive patients enrolled from the Multiple Sclerosis Centers of the 'Amedeo Avogadro' University of Eastern Piedmont (Novara), the University of Milan, IRCCS Maggiore Policlinico Hospital (Milan), the Don Gnocchi Institute (Milan), the Santa Croce Hospital (Cuneo), the University of Rome 'La Sapienza', S Andrea Hospital (Rome) and the University of Bari (Bari). Their clinical and demographic features were similar to those of other series.[43,44] Controls were consecutive Italian donors obtained from the transfusion services of the respective hospitals. Patients and controls were unrelated, Caucasian and Italian, matched for age and gender, and analyzed as follows:[45]

1. RR: Occurrence of exacerbations, each lasting at least 24 h and separated by at least 1 month of inactivity, with full recovery or sequelae ($n=852$).
2. PP: Steady worsening of symptoms and signs from onset for at least 6 months, whether superimposed with relapses or not, with occasional plateau and temporary minor improvements ($n=92$).
3. SP: Initial RR course followed by steady worsening of symptoms and signs for at least 6 months, whether superimposed with relapses or not, with minor remissions and plateaux ($n=212$).

Progression of disability was assessed with the MSSS.[46] In RR patients, MSSS score was assessed in remission phase.

All patients gave their informed consent according to the Declaration of Helsinki.[47] The research was approved by the Novara ethical committee.

Amplification of PRF1 and mutation detection

Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) using standard methods and exons 2 and 3 of the *PRF1*-coding region were amplified in standard PCR conditions. PCR products were purified with the EXO/SAP kit (GE Healthcare, Piscataway, NJ, USA). In the first population, the entire coding region was sequenced with the ABI PRISMR BigDye Terminator kit (Applied Biosystems, Foster City, CA, USA) on an automatic sequencer (Applied Biosystems 3100 Genetic Analyser) according to the manufacturer's instructions. Figure 1 shows primers used for amplification, sequencing and typing. Briefly, exon 2 was amplified with primers A + B (755 bp) and sequenced with the same oligonucleotides. Exon 3 was amplified with C + D (1289 bp) and sequenced with these and with two additional internal primers (E and F). In the second population, the +272 C/T (A91V) variation was typed by sequencing (233 patients and 548 controls) or by the TaqMan 5′-allelic discrimination assay (733 patients and 972 controls; Applied Biosystems). Allelic-specific primers and probes used for discrimination have been previously described.[20] Genotyping of each sample was automatically attributed by the SDS 1.3 software for allelic discrimination. Similar results were obtained in patients typed by the two methods. All variations were confirmed twice by sequencing independent DNA samples. The genotypic distribution of the variation did not deviate significantly from the Hardy–Weinberg equilibrium in any group.

Allele-specific PCR

The wild-type (91A) and mutant (91V) alleles were separately amplified using specific PCR amplification of genomic DNA (forward primer: G or H; reverse primer: D). PCR products were typed for P355P and R357W by sequencing with the ABI PRISMR BigDye Terminator kit on the 3100 Genetic Analyser using the internal primer E.

HLADRB1 typing

Patients and controls were specifically typed for DRB1*1501 allele as previously described.[48]

Cytotoxicity assays

Natural killer activity of PBMC was assessed by a standard 4 h ^{51}Cr-release assay with K562 cells as the target. Results are expressed as specific lysis % calculated as follows: (sample ^{51}Cr release−spontaneous release)/(maximal release−spontaneous release) × 100.

Statistical analysis

Phenotype frequencies were calculated as the number of individuals carrying an allele (either homozygotes or heterozygotes) divided by the total number of individuals.

Allelic and phenotype frequencies were compared with the χ^2-test with the Yates correction. All *P*-values are two-tailed and the significance cutoff was $P<0.05$. Putative effect of the variation on splicing sites was evaluated using the SpliceView program on the WebGene website (http://www.itb.cnr.it/sun/webgene/) and the ESEfinder scoring matrix (http://www.rulai.cshl.edu/tools/ESE). Putative functional significance of the missense variations was evaluated with the PolyPhen program (http://genetics.bwh.harvard.edu/pph).

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Disclosure

The authors report no conflict of interest.

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Expression of functional NK_1 receptors in human alveolar macrophages: superoxide anion production, cytokine release and involvement of NF-κB pathway

[1]Claudio Bardelli, [1]Gabriele Gunella, [2]Federica Varsaldi, [3]Pietro Balbo, [1]Elisa Del Boca, [1]Ilaria Seren Bernardone, [1]Angela Amoruso & *,[1,4]Sandra Brunelleschi

[1]Department of Medical Sciences, School of Medicine, University of Piemonte Orientale 'A. Avogadro', Via Solaroli, 17, 28100 Novara, Italy; [2]Department of Chemistry, Food, Pharmaceutical and Pharmacological Science (DISCAFF), University of Piemonte Orientale 'A. Avogadro', Novara, Italy; [3]Azienda Ospedaliera 'Maggiore della Carità', Novara, Italy and [4]IRCAD (Interdisciplinary Research Center on Autoimmune Diseases), University of Piemonte Orientale 'A. Avogadro', Novara, Italy

1 Substance P (SP) is deeply involved in lung pathophysiology and plays a key role in the modulation of inflammatory-immune processes. We previously demonstrated that SP activates guinea-pig alveolar macrophages (AMs) and human monocytes, but a careful examination of its effects on human AMs is still scarce.

2 This study was undertaken to establish the role of SP in human AM isolated from healthy smokers and non-smokers, by evaluating the presence of tachykinin NK_1 receptors (NK-1R) and SP's ability to induce superoxide anion (O_2^-) production and cytokine release, as well as activation of the nuclear factor-κB (NF-κB) pathway.

3 By Western blot analysis and immunofluorescence, we demonstrate that authentic NK-1R are present on human AMs, a three-fold enhanced expression being observed in healthy smokers. These NK-1R are functional, as SP and NK_1 agonists dose-dependently induce O_2^- production and cytokine release. In AMs from healthy smokers, SP evokes an enhanced respiratory burst and a significantly increased release of tumor necrosis factor-α as compared to healthy non-smokers, but has inconsistent effects on IL-10 release. The NK_1 selective antagonist CP 96,345 ((2*S*,3*S*)-*cis*-2-diphenylmethyl-*N*[(2-methoxyphenyl)-methyl]-1-azabicyclo-octan-3-amine)) competitively antagonized SP-induced effects.

4 SP activates the transcription factor NF-κB, a three-fold increased nuclear translocation being observed in AMs from healthy smokers. This effect is receptor-mediated, as it is reproduced by the NK_1 selective agonist [$Sar^9Met(O_2)^{11}$]SP and reverted by CP 96,345.

5 These results clearly indicate that human AMs possess functional NK-1R on their surface, which are upregulated in healthy smokers, providing new insights on the mechanisms involved in tobacco smoke toxicity.

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Keywords: Substance P; human alveolar macrophages; NK_1 receptor; NF-κB activation; respiratory burst; cytokine release; TNF-α; IL-1β; IL-10

Abbreviations: AMs, alveolar macrophages; CP96,345, (2*S*,3*S*)-*cis*-2-diphenylmethyl-*N*[(2-methoxyphenyl)-methyl]-1-azabicyclo-octan-3-amine); GR82334, ([D-Pro^9(spiro-gamma-lactam)Leu^{10},Trp^{11}]physalaemin(1–11)); GR71251, ([Pro^9(s-piro-gamma-lactam) Leu^{10},Trp^{11}]SP); NF-κB, nuclear factor-κB; NK-1R, NK_1 receptor; O_2^-, superoxide anion; PMA, phorbol 12-myristate 13-acetate; SP, substance P

Introduction

The neuropeptide substance P (SP), a member of the tachykinin receptor family, is involved in many physiological processes, including nociception, vasodilation, exocrine and endocrine gland secretion, smooth muscle contraction, cell proliferation, and largely contributes to the local control of the immune responses (Severini *et al*., 2002). It induces lymphocyte proliferation (Payan *et al*., 1983), enhances immunoglobulin production by cloned B lymphoma cells (Pascual *et al*., 1991), degranulates rat mast cells (Mousli *et al*., 1989), modulates eosinophil and neutrophil activity (Brunelleschi *et al*., 1991; Iwamoto *et al*., 1993), stimulates human peripheral monocytes to produce inflammatory cytokines including IL-1, IL-6, IL-12 and tumor necrosis factor-α (TNF-α) (Lotz *et al*., 1988; Lavagno *et al*., 2001).

By using natural tachykinins and selective receptor agonists and antagonists, we previously demonstrated that guinea-pig alveolar macrophages (AMs) possess NK_1 receptors (NK-1R) and NK_2 receptors, their stimulation leading to superoxide anion (O_2^-) production and eicosanoid release, and that ovalbumin-sensitized AMs demonstrate an enhanced responsiveness to NK-2R stimulation (Brunelleschi *et al*., 1990; 1992). We also showed that SP, as well as neurokinin A (NKA)

*Author for correspondence at: Department of Medical Sciences, School of Medicine, University of Piemonte Orientale 'A. Avogadro', Via Solaroli, 17, 28100 Novara, Italy;
E-mail: sandra.brunelleschi@med.unipmn.it
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and the selective NK_2 receptor agonist [β-Ala^8]-NKA(4–10), induces O_2^- production in AMs obtained from patients with active sarcoidosis (Brunelleschi *et al*., 1996).

The biological responses to SP are mediated by the G protein-coupled tachykinin NK-1R, although SP can also bind, with lower affinity, NK_2 and NK_3 tachykinin receptors (Severini *et al*., 2002; Pennefather *et al*., 2004). The presence of NK_1 receptors (NK-1R) on monocyte/macrophages has been demonstrated by evaluating the effects of selective receptor agonists and antagonists on functional parameters (for example, Brunelleschi *et al*., 1990; 1992; 1998) and/or by molecular biology and protein chemistry techniques. RT–PCR and *in situ* hybridization have been used to identify NK-1R mRNA expression in monocytes and macrophages (Ho *et al*., 1997; Germonpre *et al*., 1999), but little is known about NK_1 expression at the protein level. Marriott & Bost (2000) and Simeonidis *et al*. (2003) demonstrated the presence of NK-1R protein in murine peritoneal macrophages and THP-1 cells, respectively, but, to our knowledge, nobody has investigated this possibility in human AMs. Recent evidence indicates that NK-1R gene expression in THP-1 cells is increased after exposure to IL-1β and TNF-α: this effect is mediated by the transcription factor NF-κB, which binds to the promoter region of the NK-1R gene and so regulates its expression (Simeonidis *et al*., 2003).

In resting cells, nuclear factor-κB (NF-κB) is retained in the cytoplasm through an association with inhibitory proteins of the IκB family, which mask the nuclear localization signal (Baldwin, 1996). Upon stimulation, IκBα is phosphorylated, ubiquitinylated and degraded, thus allowing NF-κB to translocate to the nucleus. Once NF-κB enters the nucleus, it binds to the promoter region of various genes and induces their transcription (Baldwin, 1996). SP specifically activates NF-κB pathway in cells of the monocyte/macrophage lineage, for example, human astrocytoma cells, murine peritoneal macrophages and dendritic cells (Lieb *et al*., 1997; Marriott *et al*., 2000), but no information are available concerning human AMs.

The present study was undertaken to establish the role of tachykinin NK-1R in human AMs isolated from healthy smokers and non-smokers. We demonstrate the presence of authentic NK-1R, as determined by Western blot analysis and immunofluorescence, and indicate that the NK-1R expressed in human AM are functional, as demonstrated by the ability of SP to evoke O_2^- production and cytokine release. We also present direct evidence that SP activates the transcription factor NF-κB pathway, so providing new insights on the mechanisms involved in neuropeptidergic control of AM responsiveness.

Methods

Study population

This study and the research protocol were approved by the local Ethical Committee. A total of 25 individuals, 15 male and 10 female subjects, aged between 28 and 76 years, 13 smokers and 12 non-smokers, were studied. The characteristics and smoking history of the study population are presented in Table 1, Results section. None of the subjects received medical therapy at the time of the study. Broncho-alveolar lavage (BAL) was mainly performed for diagnostic purposes to have a further validation/confirmation of the suspected disease; healthy subjects were individuals who had no history of cardiopulmonary disease or other chronic disease, no diagnosed lung diseases and were not on medication. In a few cases, the attribution of a 'healthy' subject to the category was done after the BAL procedure.

Table 1 Study population

Subject	*Sex (F or M)*	*Age (years)*	*Smoker*	*Number of cigarette day^{-1}*	*Years on smoke*
1	F	51	Yes	20	25
2	M	54	Yes	12	20
3	F	43	No	—	—
4	M	63	Yes	15	40
5	M	50	No	—	—
6	F	28	Yes	10	10
7	M	43	Yes	30	18
8	M	50	No	—	—
9	M	46	No	—	—
10	F	29	No	—	—
11	M	37	Yes	20	15
12	F	35	No	—	—
13	M	69	No	—	—
14	F	56	Yes	25	32
15	M	48	No	—	—
16	M	62	Yes	16	34
17	F	70	No	—	—
18	F	45	Yes	20	15
19	M	39	No	—	—
20	M	45	Yes	35	20
21	M	55	No	—	—
22	F	76	No	—	—
23	F	33	Yes	12	8
24	M	67	Yes	10	30
25	M	58	Yes	12	33

Isolation of human AMs from BAL

AMs were isolated from BAL as described (Brunelleschi *et al*., 1996). After informed consent was obtained from each patient and pretreatment with parenteral atropine sulphate (0.5 mg), airways were anaesthetized with 2% lidocaine. A fiberoptic bronchoscope was advanced and wedged into the middle lobe under direct visualization. Lavage was carried out with 140–200 ml of prewarmed (37°C) sterile saline solution in 20-ml aliquots with immediate gentle vacuum (syringe) aspiration after each injection. The fluid so obtained was filtered through two layers of sterile surgical gauze and centrifuged ($400 \times g$, 30 min). The whole BAL pellet was washed twice in phosphate-buffered salt solution (PBS), resuspended in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS), 2 mM glutamine, 10 mM Hepes, 50 μg ml^{-1} streptomycin and 5 U ml^{-1} penicillin, and plated in six-well tissue culture plates (35 mm diameter; Costar, U.K.). After 2 h at 37°C in humidified 5% CO_2 atmosphere, nonadherent cells (mainly lymphocytes) were gently removed and AMs were used for the experiments. Total cell count and viability evaluation (Trypan blue dye exclusion test, always >98%) were performed on a Burker haemocytometer. Differential cell count was carried out on Diff-Quick (Don Baxter)-stained cytospin smears, counting at least 400 cells. The adherent cell population was >99% AM. Phenotypical analysis was carried out on cytocentrifuge (Cytospin, U.K.; 500 r.p.m., 10 min) slides by

employing leukocyte-specific monoclonal antibodies for CD68, CD14 and HLA-DR (from Becton Dickinson, U.K.).

O_2^- production in AMs

Adherent AMs ($0.4–1 \times 10^6$ cells plate^{-1}) were washed twice with PBS, incubated in RPMI 1640 medium (without phenol red, no antibiotics and no FCS) and challenged with increasing concentrations of tachykinins for 30 min. SP is the major endogenous ligand for NK-1R, [Sar^{9}Met(O$_2$)^{11}]SP and Pro^{9}SP are selective NK$_1$ agonists. In the experiments with the NK-1R antagonists CP 96,345, GR82334 ([D-Pro^9(spiro-gamma-lactam)Leu^{10},Trp^{11}]physalaemin(1-11)) and GR71251 (([Pro^9(spiro-gamma-lactam) Leu^{10},Trp^{11}]SP)), AMs were pre-incubated for 15 min with these drugs and then challenged with tachykinins. The effects of tachykinins were compared with those evoked by phorbol 12-mirystate 13-acetate (PMA), a standard stimulus acting as a direct protein kinase C activator. The O_2^- production was evaluated by the superoxide dismutase (SOD)-inhibitable cytochrome *c* reduction, the absorbance changes being recorded at 550 nm in a Beckman DU 650 spectrophotometer. O_2^- production was expressed as nmol cytochrome *c* reduced/10^6 cells/30 min, using an extinction coefficient of 21.1 mM (Brunelleschi *et al*., 2001). To avoid interference with spectrophotometrical recordings of O_2^- production, AMs were incubated with RPMI 1640 without phenol red. Experiments were performed in duplicate or triplicate; control values (e.g., basal O_2^- production in the absence of stimuli) were subtracted from all determinations.

Release of TNF-α and other cytokines from AMs

Adherent AMs were challenged with the selected stimuli (SP, NK$_1$ selective agonists, PMA) for 24 h at 37°C to ensure maximal cytokine release. Supernatants were collected and stored at −20°C. TNF-α, IL-1β and IL-10 (the latter was evaluated as the most important anti-inflammatory cytokine) in the samples were measured using enzyme-linked immunoassay kit (Pelikine Compact™ human ELISA kit). The measurements were performed according to the manufacturer's instructions. The minimum detectable concentrations of human TNF-α, IL-1β and IL-10 were 1.4, 1.5 and 1.3 pg ml^{-1}, respectively. No crossreactivity was observed with any other known cytokine. Control values (e.g., cytokine release from untreated, unstimulated cells) were subtracted from all determinations. Results are expressed in pg ml^{-1}.

Immunofluorescence for NK-1R in AMs

Human AMs were cultured onto gelatin-coated glass slides. The cells were fixed in ice-cold 4% paraformaldahyde (20 min), washed twice with PBS, permeabilized with 0.5% Triton X-100 (15 min, 25°C), washed twice with PBS, and blocked with 10% FCS, 2% BSA, 1% glycine, 0.5% Triton X-100 in PBS (1 h, 25°C). The cells were then incubated in the presence of a rabbit antibody directed against the human NK-1R (Santa Cruz Biotechnology, U.S.A.) at a dilution of 1 : 120 in PBS overnight at 4°C. After washing, FITC-conjugated anti-rabbit immunoglobulins (1 : 30) (Dako Cytomation, Milan, Italy) were added (2 h, 25°C). After another washing with PBS, nuclear staining was performed using Hoechst 33258 (0.8 μg ml^{-1}, 1 h, 37°C) (Sigma-Aldrich, Milan, Italy). Fluorescence was visualized using 100-fold magnification.

Western blotting of the tachykinin NK-1R in AMs

Subconfluent AMs (10×10^6 cells) were washed twice with ice-cold PBS and lysed with 1 ml RIPA buffer (1% Triton X-100, 1% sodium deoxycolate, 0.1% SDS, 50 mM Hepes pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM NaF) containing 1 mM Na_3VO_4 and protease inhibitors (10 μg ml^{-1} aprotinin, 10 μg ml^{-1} pepstatin, 50 μg ml^{-1} leupeptin, 1 mM phenylmethylsulphonyl fluoride-PMSF). Cells were placed on ice for 20 min and scraped. Cell lysates were sonicated on ice four times for 5 s each, cleared by centrifugation at $15{,}000 \times g$ for 10 min at 4°C and the supernatants (cell lysates) transferred into a new tube. If necessary, cell lysates were stored at −80°C. About 30 μg total extracts were separated on 8–10% SDS–PAGE and transferred to nitrocellulose filters (Protran, Perkin-Elmer Life Sciences, Boston, MA U.S.A.). Nonspecific binding sites on membrane were blocked at room temperature for 1 h in TBS-5% BSA nitrocellulose filters. Filters were probed with a commercial anti-human NK-1R antibody (NK-1R (H-83): sc-15323, Santa Cruz Biotechnology, U.S.A.; a rabbit polyclonal antibody mapping at the C-terminus of the human NK$_1$ receptor) (1 : 200 in TBS-5% BSA) for 2 h at room temperature. Proteins were visualized by using ECL Western blotting detection reagents (Perkin-Elmer Life Science, Boston, MA, U.S.A.). Quantification of Western blots was performed by densitometry using 'Quantity One, 1-D Analysis' software (Bio-Rad, U.S.A.) and expressed as intensity data units.

Evaluation of NF-κB activation

The activation of NF-κB induced by SP, NK$_1$ agonists or PMA was evaluated by measuring the nuclear migration (by electrophoretic mobility shift assay (EMSA)) as well as the nuclear content of p50 and p65 subunits (by ELISA and Western blot). The methods we used are detailed below.

Preparation of nuclear and cytosolic cellular fractions

After challenge with stimuli, AMs (about 5×10^6 cells) were washed with ice-cold PBS, scraped and centrifuged at $1000 \times g$ for 5 min at 4°C. The cell pellet was resuspended in 300 μl of lysis buffer (10 mM Hepes, pH 7.6, 60 mM KCl, 1 mM EDTA, 1 mM PMSF, 1 mM dithiothreitol, 10 μl ml^{-1} protease cocktail inhibitors) and incubated on ice for 15 min. At the end of this incubation, 20 μl of 10% NP-40 was added and the tube vortexed for 10 s. After centrifugation at $13{,}000 \times g$ for 1 min at 4°C, supernatants (cytosolic fractions) were collected and stored at −80°C, whereas the pellets were further processed to obtain nuclear extracts. The pellets were resuspended in extraction buffer (20 mM Tris–HCl, pH 8, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM PMSF, 0.2 mM EDTA, 10 μl ml^{-1} protease cocktail inhibitors, glycerol 25% v v^{-1}) and incubated for 30 min at 4°C. Nuclear proteins were isolated by centrifugation at $13{,}000 \times g$ for 15 min. The supernatant was aliquoted and stored at −80°C until used for EMSA or p50/p60 ELISA assays. Protein concentrations were determined by using a protein assay (Bio-Rad, U.S.A.).

EMSA of NF-κB

Nuclear extracts (5 μg) were incubated with 2 μg poly (dI-dC) and the γ [^{32}P]ATP-labelled oligonucleotide probe (100,000–150,000 c.p.m.; Promega) in binding buffer (50% glycerol, 10 mM Tris–HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 1 mM dithiothreitol) in a final volume of 20 μl for 30 min at room temperature. The NF-κB consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was obtained from Promega. The nucleotide–protein complex was separated on a 5% polyacrylamide gel in 0.5 × TBE buffer (100 mM Tris–HCl, 100 μM boric acid, 2 mM EDTA) at 150 V on ice. The gel was dried and radioactive bands were detected by autoradiography.

p50 and p65/RelA assays

Nuclear extracts were prepared as described above and evaluated for the presence of p50 and p65/RelA subunits using Trans AM™ NF-κB p50 Chemi and NF-κB p65 Chemi Transcription Factor Assay kits (Active Motif Europe, Belgium), according to the manufacturer's instructions. Briefly, an equal amount (1 μg) of nuclear lysate was added to incubation wells precoated with an oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) sequence, the active form of NF-κB contained in the cell extract specifically binding to this oligonucleotide. Sometimes, the cytosolic content of both subunits was also measured. These assay kits specifically detected bound NF-κB p65 or p50 subunits in human extracts; activities of p50 and p65 were measured by a Rosys Anthos Lucy 1 luminometer and results are expressed as RLU (relative luminescence unit). In some cases, the nuclear extracts (5 μg protein, for each sample) were also used to evaluate p50 and p65 subunits by Western blot. For these assays, commercial antibodies (anti-NF-κB p50: ab 7971 and anti-NF-κB p65: ab 7970) were obtained from Abcam (U.K.). Nuclear extracts were challenged for 2 h at room temperature with the antibody at a final concentration of 1 μg ml^{-1}.

Drugs and analytical reagents

Substance P, selective NK_1 agonists and NK_1 antagonists were obtained from Neo-System (Strasbourg, France). The anti-NK-1R-specific antibody (NK-1R (H-83): sc-15323) was from Santa Cruz Biotechnology (U.S.A.). The anti-NF-κB p50 and anti-NF-κB p65 antibodies were obtained from Abcam (U.K.). PBS, RPMI 1640 (with or without phenol red), BSA, glutamine, Hepes, streptomycin, penicillin, PMA, ethanol, SOD, cytochrome *c*, Na-deoxycholate, NaCl, EDTA, protease cocktail inhibitors (aprotinin 0.3 μM, bestatin 130 μM, leupeptin 1 μM), bromophenol blue, glycine, glycerol, methanol and Tween 20 were obtained from Sigma (Milwaukee, WI, U.S.A.). Poly(dI-dC) were obtained from Pharmacia (Uppsala, Sweden). Triton X-100 and β-mercaptoethanol were from Fluka (Buchs, Switzerland); PMSF was from Promega (Madison, WI, U.S.A.). SDS and DMSO were from Merck (Darmstadt, Germany). BCA Protein Assay Reagent kit was from Pierce (Rockford, IL, U.S.A.). Nitrocellulose filters (Hybond) and the enhanced chemiluminescence system were from Amersham (Buckinghamshire, U.K.). Tissue-culture plates were purchased from Costar Ltd (Buckinghamshire, U.K.). All cell culture reagents, with the exception of fetal bovine serum, were endotoxin-free according to details provided by the manufacturer. Fetal bovine serum (lot 40G3410 K, containing <10 EU ml^{-1}) was from Life Technologies Inc. (Rockville, U.S.A.). TNF-α, IL-1β and IL-10 immunoassay kit was obtained from CLB/Sanquin, Central Laboratory of the Netherlands Red Cross (Netherlands). Gel shift assay Core system and all the reagents for NF-κB EMSA were from Promega Corporation (St Louis, CA, U.S.A.).

Data and statistical analysis

Data are mean ± s.e.m. of duplicate determinations of '*n*' independent experiments. Concentration–response curves for SP and NK_1 agonists were constructed and EC_{50} values were interpolated from curves of best-fit. When required, statistical evaluation was performed by Student's *t* test.

Results

Study population, BAL and phenotype of AMs

In all, 25 individuals, 15 male and 10 female subjects (mean age = 50.2 ± 2.7 years; mean age of male and female subjects: 55 ± 3.7 and 44.5 ± 4.3 years, respectively, $P = 0.07$), were studied. A total of 13 (eight male and five female subjects) were smokers and 12 (seven male and five female subjects) were non-smokers; mean age of smokers (49.4 ± 3.3 years; $n = 13$) and non-smokers (50.8 ± 4 years, $n = 12$) being very similar. The characteristics and smoking history of the study population are listed in Table 1. None of the subjects received medical therapy at the time of the study. Total and differential cell counts in BAL and phenotype of AMs from smokers and non-smokers are presented in Table 2. As expected, a significant ($P < 0.05$) increase in the total cell number in BAL (with no significant differences in differential cell counts) was observed in smokers as compared to non-smokers. The great majority of AMs (96 ± 1%) in healthy smokers was CD68 + and a high percentage (86 ± 1 and 66 ± 3%, respectively) of AM expressed also HLA-DR and CD14. As known, CD68 expression is related to the presence of AM involved in the oxidative burst, CD14 expression is related to cytokine production by LPS receptor, whereas HLA-DR is related to antigen presentation. The expression CD14 and CD68 was significantly ($P < 0.05$) higher in AMs collected from healthy smokers as compared to healthy non-smokers (Table 2).

NK-1R expression in human AMs

We examined the expression pattern of the NK-1R gene products in human AMs. We collected AMs from healthy smokers and healthy non-smokers undergoing BAL for diagnostic procedures, after their informed consent. First, we performed immunofluorescence assays in AMs isolated from healthy non-smokers and observed the expression of the NK-1R protein localized primarily at the cell surface (Figure 1). A phase contrast photomicrograph of AM is shown in Figure 1a. Immunofluorescence with anti-NK-1R antibody and FITC-conjugated anti-rabbit antibody reveals a green colour (Figure 1b), nuclear staining with Hoechst 33258 (0.8 μg ml^{-1}, 30 min, 37°C) is depicted in blue (Figure 1c),

Table 2 Total and differential cell count in BAL and AM phenotype

Subjects	*Total cell* ml^{-1} *BAL*	*AMS (%)*	*Lympho (%)*	*PMNs (%)*	*CD68+ (%)*	*HLA-DR+ (%)*	*CD14+ (%)*
Smokers ($n=13$)	390.000±6.000	90.8±1.9	8.2±2	1±0.5	96±1	86±1	66±3
Non-smokers ($n=12$)	139.000±5.100*	90.6±1	8.6±1	0.2±0.1	83±1*	84±2	50±2*

Data are given as total cell number ml^{-1} BAL and percentage of total cell population (differential) in BAL. AMs = alveolar macrophages; Lympho = alveolar lymphocytes; PMNs = alveolar neutrophils. The AM phenotype was evaluated by measuring CD68, CD14 and HLA-DR: positive cells are expressed as percentage of total AMs.
*Denotes $P<0.05$ vs smokers.

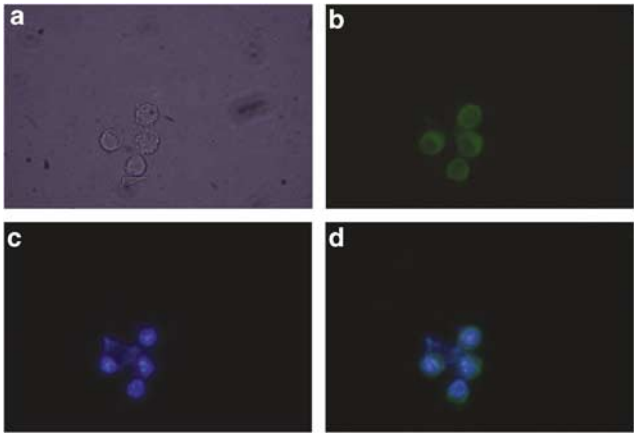

Figure 1 Immunocytochemical analysis of the expression and location of NK-1R in human AMs. (a) Phase contrast. (b) Immunofluorescence of anti-NK-1R polyclonal Ab followed by FITC-conjugated anti-rabbit immunoglubulins (green). (c) Nuclear staining with Hoechst 33258 (blue). (d) Merge of B and C. Fluorescence was visualized by a vertical fluorescence microscopy (100-fold magnification; Eclipse E600, Nikon, Tokyo, Japan).

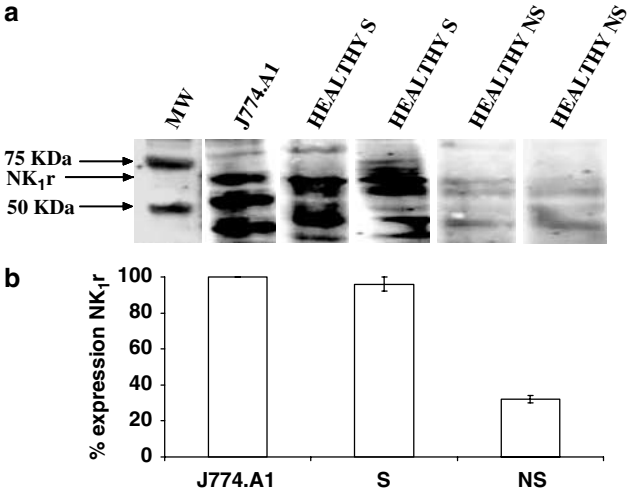

Figure 2 NK-1R expression in human AMs from healthy smokers (S) and non-smokers (NS). In (a), Western blot analysis of NK-1R in AM extracts of two smokers and two non-smokers. The blots are assembled from different single experiments in which AMs from S or NS have been evaluated. As a positive control for the presence of NK-1R, the macrophage cell line J774.A1 was used (for clarity, only one blot of J774.A1 cells, representative of seven others, is shown). The same protein amount (30 μg) was used in each experiment with AMs from smokers and non-smokers. An arrowhead indicates the 68-kDa band corresponding to the receptor. The migration of protein standards of known sizes is shown on the left. In (b), quantitative evaluation of NK-1R expression by densitometry. Intensity of the specific band of NK-1R in the macrophage J774.A1 cells amounted to 8710±250 intensity units (means±s.e.m. of eight experiments) and was taken as 100%. Results are expressed as % expression of the positive control; mean + s.e.m. of four experiments for S and NS.

and merge of both visualizes NK-1R on AM surface (Figure 1d).

We next performed Western blot in AMs isolated from healthy smokers and non-smokers to evaluate NK-1R expression at the protein level (Figure 2). AMs were obtained from four healthy smokers (subjects 1, 2, 14 and 20) and four healthy non-smokers (subjects 3, 5, 10 and 21); the experiments were performed separately (by using the same protein amount, 30 μg, for each individual assay) and were always compared with the NK-1R expression in the positive control, the cultured J774.A1 cells (a murine macrophage cell line). As shown in Figure 2a, Western blot analysis of J774.A1 cells, AMs from two healthy smokers (subjects 1 and 2) and two non-smokers (subjects 3 and 5), probed with the polyclonal anti-NK-1R antibody, reveals three prominent bands of 68, 53 and 42 kDa, respectively (Figure 2a). According to manufacturer's instructions, the commercial polyclonal antibody we used detected a protein of about 68 kDa. As known, NK-1R possess different sites for acetylation and phosphorylation and may be present as truncated forms (Li *et al*., 1997; Page & Bell, 2002; Caberlotto *et al*., 2003). In our experiments, we always observed a positive band at 68 kDa (as indicated by the manufacturer) and two bands of 53 and 42 kDa (Figure 2a). Such observations are in keeping with previous reports in which other authors, by using noncommercial monoclonal NK-1R antibodies, detected a 46 kDa protein in human antral tissue (Smith *et al*., 2000), a molecular mass band of 53 kDa in the monocyte/macrophage THP-1 cells (Simeonidis *et al*., 2003) or a 42 kDa protein in murine peritoneal macrophages and murine microglia (Marriott & Bost, 2000; Rasley *et al*., 2002).

Interestingly, densitometric evaluation of NK-1R expression revealed that AMs collected from healthy smokers demonstrated a >3-fold increase as compared to AMs isolated from healthy non-smokers (Figure 2b). The intensity of the specific band of 68 kDa in the positive control, the J774.A1 cell line, amounted to 8710±250 intensity units ($n=8$) and was taken as 100%. NK-1R expression in AMs from healthy non-smokers was 32±1.5% ($n=4$), whereas NK-1R expression in AMs from healthy smokers amounted to 96±4% ($n=4$).

NK-1R are functional in human AMs: O_2^- production and cytokine release

Basal values (O_2^- production from unstimulated AMs) in smokers and non-smokers were 13.5±2 ($n=6$) and 2.2±0.4

($n=5$; $P<0.01$) nmol cytochrome c reduced/10^6 AMs, respectively. These values were subtracted from those obtained after tachykinins or PMA challenge to obtain the net O_2^- production. PMA, used at 10^{-7} M (a near maximal concentration), produced 23.5 ± 2 ($n=6$) and 17 ± 0.6 ($n=5$; $P<0.05$) nmol cytochrome c reduced/10^6 AMs in smokers and non-smokers, respectively. In the concentration range 10^{-12}–10^{-6} M, SP dose-dependently evoked O_2^- production in AMs from both smokers and non-smokers, higher production being observed in smokers (Figure 3). As depicted in Figure 3, maximal activation by SP was observed at 10^{-6} M, EC_{50}s being 0.25 nM in smokers and 1 nM in non-smokers. In the presence of a cocktail of inhibitors (thiorphan, captopril and bestatin, all at 1 μM) of tachykinin degrading enzymes, SP effects were significantly enhanced (data not shown). The metabolically stable NK-1R agonist [Sar^{9}Met(O$_2$)^{11}]SP, although less potent than SP, evoked a significant respiratory burst in AMs collected from both smokers and non-smokers, EC_{50}s being about 3 nM in smokers and 10 nM in non-smokers (Figure 3). Pro^{9}SP, the other NK$_1$ selective agonist we used, acted dose-dependently, although less active and potent than SP or [Sar^{9}Met(O$_2$)^{11}]SP (Figure 3). EC_{50}s for Pro^{9}SP were about 10 nM in smokers and 30 nM in non-smokers (Figure 3). The nonpeptide NK$_1$ selective antagonist CP 96,345 at 1 nM competitively antagonized the effects of SP and NK$_1$ selective agonists: the dose–response curve for SP was shifted to the right about two orders of magnitude (Figure 4a) as were those for [Sar^{9}Met(O$_2$)^{11}]SP (about 1.5-fold; Figure 4b) and Pro^{9}SP (one order of magnitude; not shown). GR82334, a reversible NK$_1$ antagonist devoid of histamine-releasing properties in rat mast cells (Guo *et al*., 1995), and GR71251, a selective NK$_1$ antagonist that possesses GABA-releasing actions in rat spinal cord (Hagan *et al*., 1990), at 1 μM competitively antagonized the effects of SP and NK$_1$ selective agonists in AM (data not shown).

We also evaluated the release of proinflammatory cytokines, namely TNF-α (the most abundant cytokine in AMs) and IL-1β, as well as IL-10 release (the most relevant anti-inflammatory cytokine in AMs), after challenge with tachykinins or PMA. Basal values (that is the release from

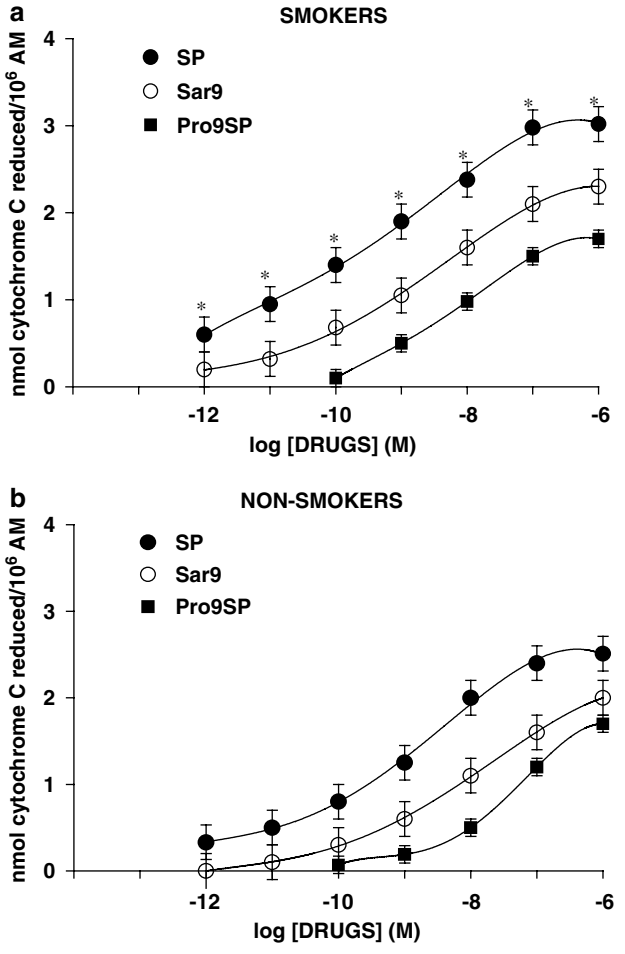

Figure 3 NK-1R agonists evoke O_2^- production in human AMs isolated from healthy smokers (a) and non-smokers (b). Cells were challenged with increasing concentrations of SP, [Sar^{9}Met(O$_2$)^{11}]SP and Pro^{9}SP for 30 min. Results are means$\pm$s.e.m. of five to six experiments in duplicate. *$P<0.05$ vs non-smokers.

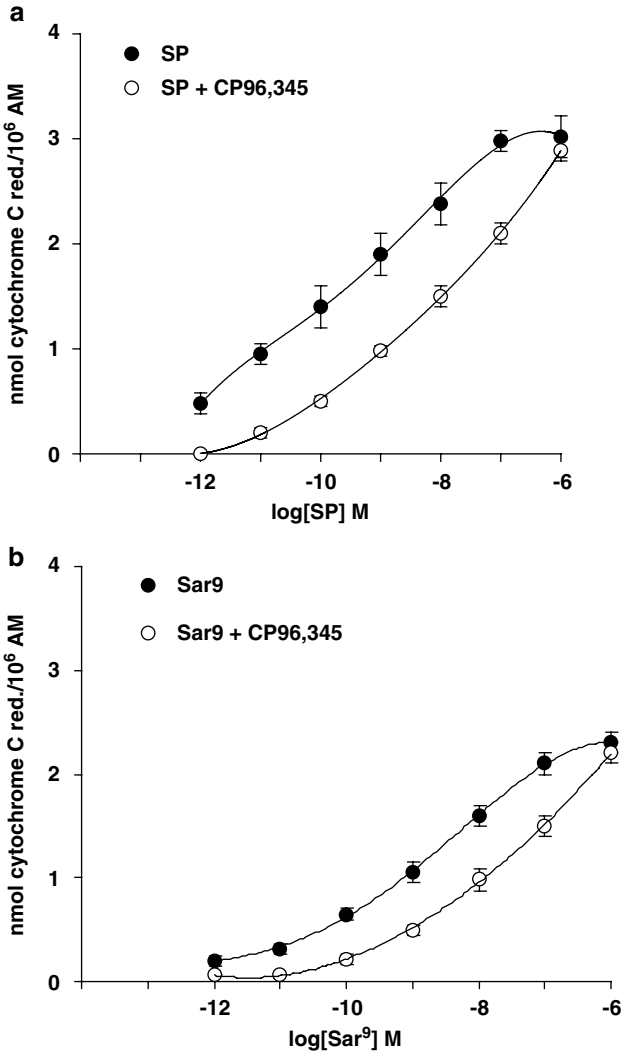

Figure 4 The NK$_1$ selective antagonist CP96,345 competitively antagonizes SP-induced O_2^- production (a) and [Sar^{9}Met(O$_2$)^{11}]SP-induced O_2^- production (b) in AMs from healthy smokers. Cells were preincubated with CP 96,345 at 1 nM for 15 min and then challenged with the NK$_1$ agonists for further 30 min. Results are means$\pm$s.e.m. of four experiments in duplicate.

Table 3 Basal release of cytokines in AMs collected from healthy smokers and non-smokers

Cytokine	*Smokers (n = 6)*	*Non-smokers (n = 6)*
TNF-α (pg ml^{-1})	42 ± 8*	11 ± 2
IL-1β (pg ml^{-1})	15 ± 4	10 ± 3
IL-10 (pg ml^{-1})	13 ± 5	15 ± 7

Values are means ± s.e.m. of experiments in triplicate. *$P < 0.05$ vs non-smokers.

unstimulated AM) were subtracted from all determinations and are listed in Table 3. As shown in Figure 5, SP (Figure 5a) and the NK$_1$ agonist [Sar^{9}Met(O$_2$)^{11}]SP (Figure 5b) dose-dependently induced TNF-α release from AMs, a significantly higher effect being observed in AMs from healthy smokers as compared to non-smokers (Figure 5a and b; $P < 0.01$). CP 96,345, tested at 1 nM, competitively antagonized SP-induced TNF-α release in smokers' AMs (Figure 5c), so confirming that this effect is mediated by NK-1R activation. Also PMA, at 10^{-7} M, induced a higher TNF-α release in AMs from smokers (535 ± 60 pg ml^{-1}; $n = 8$) as compared to non-smokers (136 ± 20 pg ml^{-1}; $n = 6$; $P < 0.05$). By evaluating IL-1β production from human AMs, we observed that SP acted dose-dependently, maximal release (about 40 pg ml^{-1}) being detected at 0.1–1 μM, with no major difference between smokers and non-smokers (Figure 6a). PMA, at 10^{-7} M, also released similar amounts of IL-1β in both smokers and non-smokers (Figure 6a). SP, in a dose-independent way, released very small amounts of the anti-inflammatory cytokine IL-10 (Figure 6b) by human AMs, a higher but not significant release being observed in non-smokers. As depicted in Figure 6, PMA 10^{-7} M did not stimulate IL-10 secretion, while inducing IL-1β release from human AMs.

SP and NK-1R agonists induce NF-κB activation

Previous observations indicate that, in different cell models, SP can induce the activation of the transcription factor NF-κB. We checked this hypothesis in human AMs by first evaluating the nuclear translocation of NF-κB by EMSA and, to ensure a better quantitative evaluation, the amounts of translocated p50 and p65 subunits by an ELISA kit. As known, although different NF-κB forms have been described, the p50–p65 heterodimer is the predominant species in many cell types (Baldwin, 1996). As a positive control for the detection of NF-κB activation, human AMs were stimulated by PMA, as this agent has previously been demonstrated to induce NF-κB nuclear translocation in human monocytes (Lavagno *et al.*, 2004). To investigate the time- and dose-dependent effects of SP, AMs from healthy non-smokers (Figure 7a) and smokers (Figure 7b) were challenged with two different concentrations of SP (10^{-8} and 10^{-6} M) for different times (1–2 h). As reported in Figure 7, SP-induced NF-κB activation, just detectable after 1 h, was maximal at 2 h and had about the same intensity as PMA (Figure 7). Interestingly, AMs obtained from healthy smokers (Figure 7b) demonstrated a constitutively (control, unstimulated AMs, lane 7) enhanced nuclear translocation of the transcription factor NF-κB as compared to AMs isolated from non-smokers (Figure 7a, lane 7). Accordingly, PMA- and SP-induced nuclear translocation was higher in AMs from

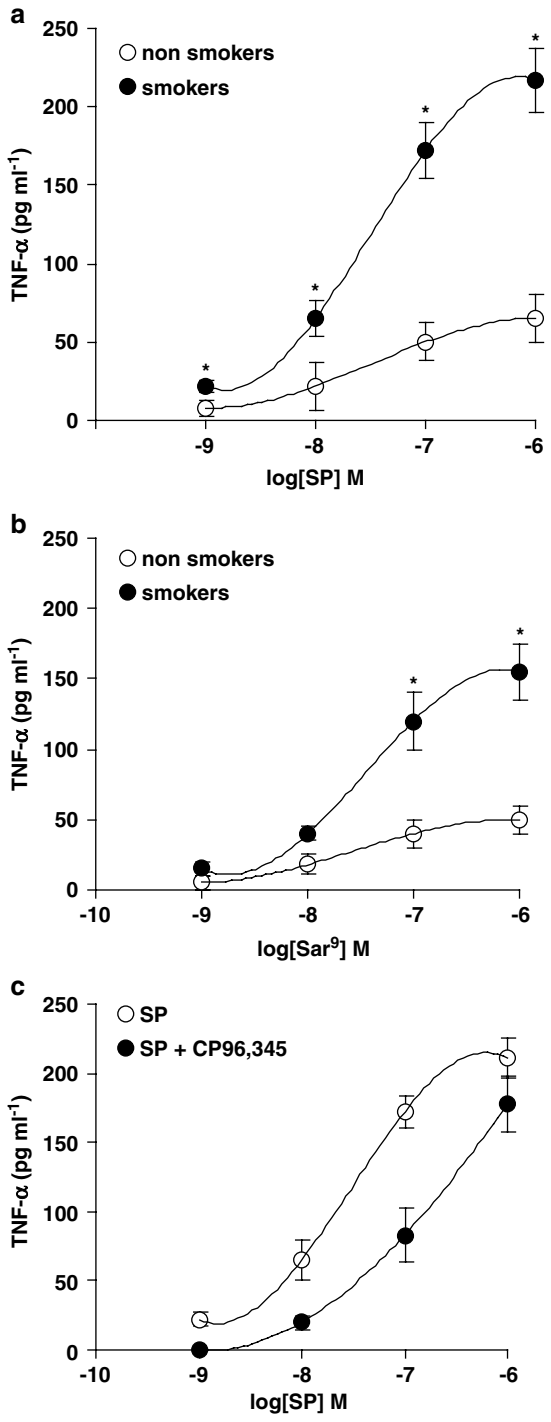

Figure 5 NK-1R stimulation induces TNF-α release in human AMs isolated from healthy smokers and healthy non-smokers. SP-induced TNF-α release in (a); [Sar^{9}Met (O$_2$)^{11}]SP-induced release in (b); reversal by CP 96,345 1 nM of SP-induced release in smokers in (c). Data are means ± s.e.m. of five to six experiments in duplicate. *$P < 0.01$ vs non-smokers. See text for further details.

smokers (Figure 7, lanes 1–2: PMA10^{-6} M, 2 and 1 h challenge, respectively; lanes 3–6: SP 10^{-6} and 10^{-8} M, 1 or 2 h challenge) as compared to non-smokers. The NK-1R antagonist CP96,345 ((2*S*,3*S*)-*cis*-2-diphenylmethyl-*N*[(2-methoxyphenyl)-methyl]-1-azabicyclo-octan-3-amine)), here evaluated at 1 μM, while not affecting *per se* PMA-stimulated translocation

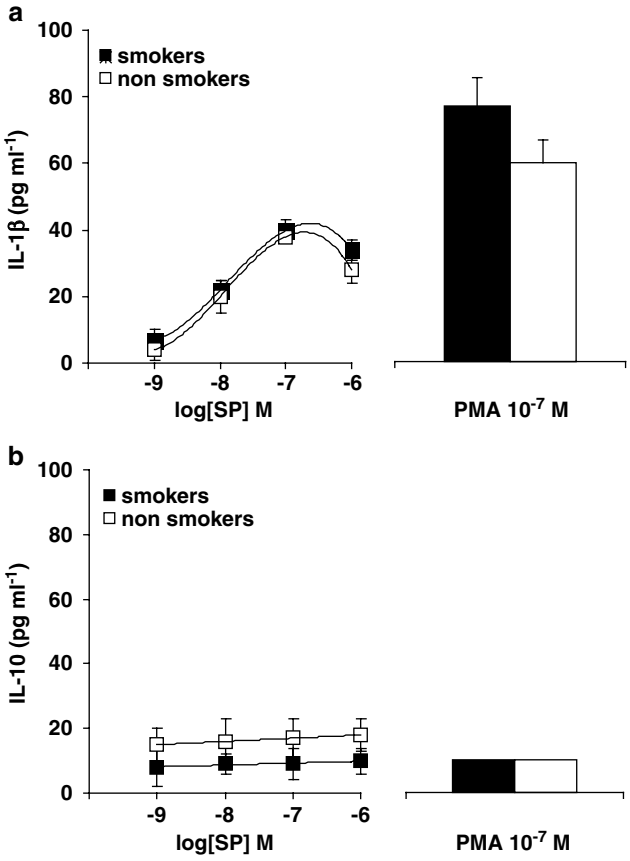

Figure 6 Effects of SP on IL-1β release (a) and IL-10 release (b) in human AMs isolated from healthy smokers and healthy non-smokers. PMA-induced release is shown for comparison. Data are means$\pm$s.e.m. of five to six experiments in duplicate.

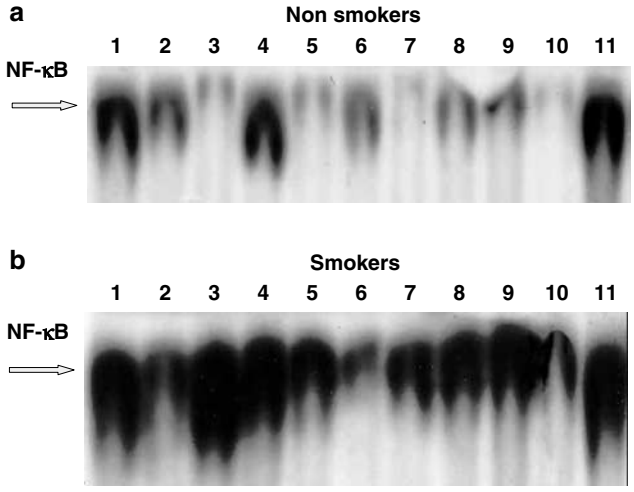

Figure 7 SP induces NF-κB activation in human AMs from healthy non-smokers (a) and healthy smokers (b) in a time- and dose-dependent manner. AMs were stimulated with SP (10^{-6} and 10^{-8} M) or PMA 10^{-6} M for 1 or 2 h, in the presence or absence of CP 96,345. The NK-1R antagonist was evaluated at 1 μM and preincubated for 15 min. Nuclear extracts (5 μg) were prepared and assayed for NF-κB activity by EMSA (see text for further details). In (a) (non-smoker): lane 1 = PMA 2 h; lane 2 = PMA 1 h; lane 3 = SP 10^{-6} M 1 h; lane 4 = SP 10^{-6} M 2 h; lane 5 = SP 10^{-8} M 1 h; lane 6 = SP 10^{-8} M 2 h; lane 7 = control, unstimulated AM; lane 8 = CP 96,345 + SP 10^{-8} M 2 h; lane 9 = CP 96.345 + SP 10^{-6} M 2 h; lane 10 = CP 96,345 alone; lane 11 = CP 96,345 + PMA 2 h. In (b) (smoker): lane 1 = PMA 2 h; lane 2 = PMA 1 h; lane 3 = SP 10^{-6} M 2 h; lane 4 = SP 10^{-6} M 1 h; lane 5 = SP 10^{-8} M 2 h; lane 6 = SP 10^{-8} M 1 h; lane 7 = control, unstimulated AM; lane 8 = CP 96,345 + SP 10^{-8} M 2 h; lane 9 = CP 96.345 + SP 10^{-6} M 2 h; lane 10 = CP 96,345 alone; lane 11 = CP 96,345 + PMA 2 h. This experiment was performed three times with similar results.

(Figure 7, lane 11) and basal constitutive activity (lane 10), potently reduced SP-induced effects (lanes 8 and 9), so confirming that SP-induced NF-κB nuclear translocation in human AMs is a receptor-mediated effect. Moreover, the NK_1 antagonist seemed to be less effective in reducing SP-induced nuclear translocation in smokers, as compared to smokers. The autoradiographs presented in Figure 7 (which are representative of two other additional experiments) are relative to AMs from smokers and non-smokers, which were obtained and contemporarily processed on the same day; the two gels presented as Figure 7a (non-smokers) and Figure 7b (smokers) were run concomitantly. Competition experiments performed with 100-fold excess unlabelled NF-κB sequence demonstrated the specificity of the induced NF-κB/DNA binding complex (not shown).

In AMs collected from healthy smokers, SP effects are reproduced, to about the same intensity, by the NK_1 agonist [$Sar^9Met(O_2)^{11}$]SP, evaluated at 10^{-8} and 10^{-6} M (Figure 8). The antagonist CP 96,345 potently reduced the effects of the NK_1 agonist, so confirming that NF-κB nuclear translocation is a receptor-mediated effect (Figure 8).

We also performed Western blot experiments to evaluate the nuclear translocation of p50 and p65 subunits of the NF-κB complex in AMs from both smokers and non-smokers. As depicted in Figure 9 (a: p50 subunit and b: p65 subunit), PMA, SP and the NK_1 agonist [$Sar^9Met(O_2)^{11}$]SP induced the translocation of both subunits, an increased p50 translocation being observed in smokers (Figure 9).

To ensure a better quantitative evaluation, we also assessed the translocation of p65 subunit and p50 subunit in AMs from both smokers and non-smokers, by using a commercially available ELISA kit. As depicted in Figure 10, SP dose-dependently induced p50 translocation (Figure 10a) and p65 translocation (Figure 10b) in AMs, a significantly ($P<0.05$) enhanced effect being observed in smokers especially for the p50 subunit. The NK_1 agonist [$Sar^9Met(O_2)^{11}$]SP, here evaluated at 1 μM, also increased p50 and p65 translocation, AMs from smokers depicting a significant enhanced p50 translocation. PMA-induced translocation is shown for comparison (Figure 10). CP 96,345 at 10^{-6} M significantly reduced SP-induced nuclear p50 and p65 translocation (not shown). Results are expressed as the nuclear/cytosolic ratio, which is the ratio between the amount of p50 (or p65) in nuclear extracts and cytosolic extracts.

Discussion

These results demonstrate that human AMs, isolated from both healthy smokers and non-smokers, possess functional NK-1R. In fact, we here report the presence of NK-1R protein (by Western blotting) as well as the ability of SP, the endogenous NK-1R ligand, to evoke O_2^- production and

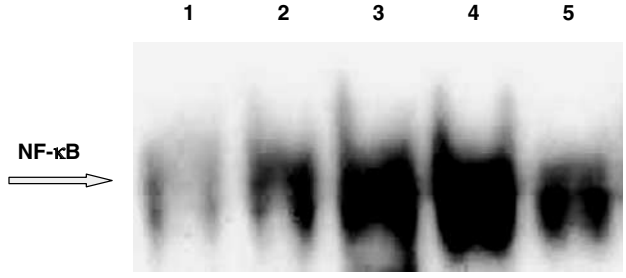

Figure 8 The NK_1 selective agonist [$Sar^9Met(O_2)^{11}$]SP induces NF-κB activation in human AMs from healthy smokers and its effects are reduced by the NK-1R antagonist CP 96,345. AMs were stimulated with [$Sar^9Met(O_2)^{11}$]SP 10^{-8} or 10^{-6} M for 2 h, in the presence or absence of CP 96,345 at 1 μM. The effects of SP 10^{-6} M are shown for comparison. Nuclear extracts (5 μg) were prepared and assayed for NF-κB activity by EMSA (see text for further details). Lane 1 = control, unstimulated AM; lane 2 = Sar^9 10^{-8} M; lane 3 = Sar^9 10^{-6} M; lane 4 = SP 10^{-6} M; lane 5 = CP 96,345 + Sar^9 10^{-6} M. This experiment was performed three times with similar results.

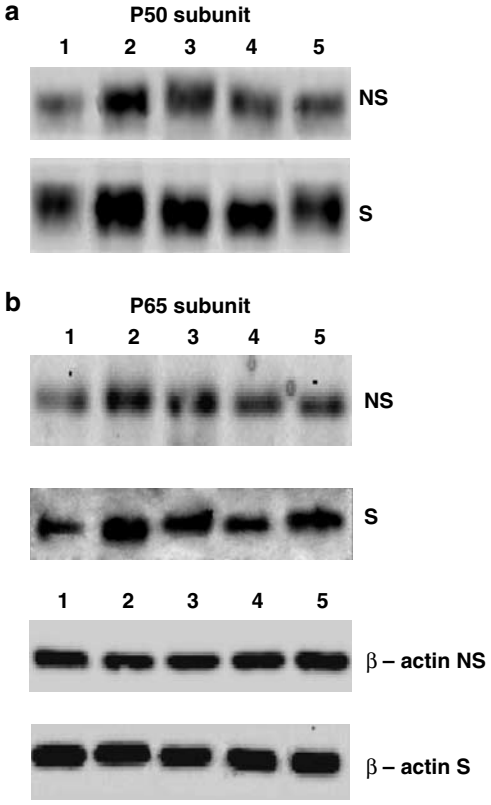

Figure 9 Western blots of p50 and p65 subunits in AMs from both non-smokers (NS) and smokers (S). The nuclear translocation of p50 is reported in (a); the translocation of p65 is reported in (b). Beta-actin is shown for comparison. Lane 1 = control; lane 2 = PMA 10^{-6} M; lane 3 = SP 10^{-6} M; lane 4 = SP 10^{-8} M; lane 5 = Sar^9 10^{-6} M.

cytokine release in AMs, as well as to induce activation of the transcription factor NF-κB. These effects are all receptor-mediated, as they are reproduced by NK_1 selective agonists and reverted by NK-1R antagonists.

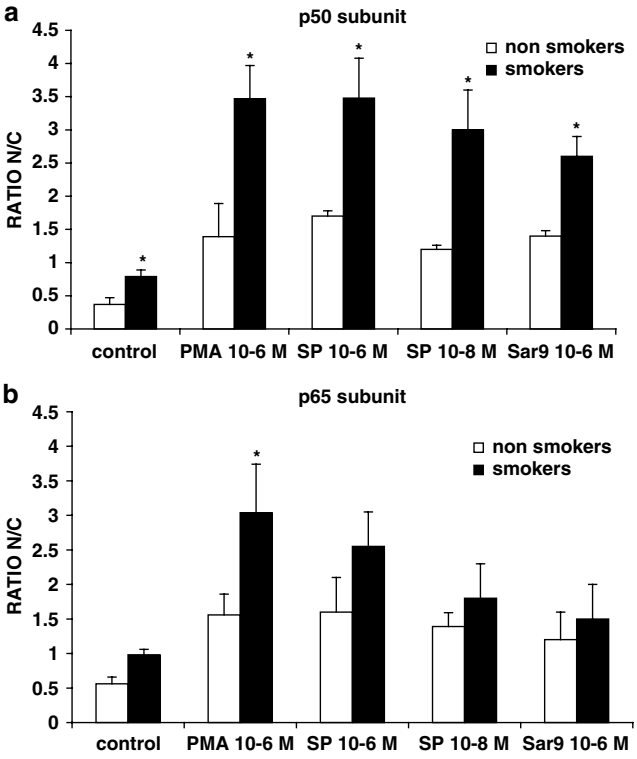

Figure 10 NK-1R stimulation induces the translocation of p50 (a) and p65 (b) subunits in human AMs from healthy smokers and healthy non-smokers. PMA-induced translocation is shown for comparison. AMs were challenged for 2 h with the stimuli; nuclear and cytosolic extracts were prepared and evaluated for their content in p50 and p65 subunits. Results are expressed as the nuclear/cytosolic ratio (ratio N/C) for both p50 and p65 subunits. Data are means ± s.e.m. of five experiments in duplicate. *$P<0.05$ vs non-smokers.

NK-1R expression has been evaluated by different authors in different cell types, by using RT–PCR technology, mainly. By this approach, human monocytes and monocyte-derived macrophages were shown to express SP and NK-1R, besides producing and releasing SP (Ho *et al*., 1997). Moreover, an NK-1R antagonist downregulated SP mRNA expression in monocyte-derived macrophages (Lai *et al*., 2002a). Lai *et al*. (2002b) quantified SP mRNA in different cells by real time RT–PCR and reported a large variability in the level of transcripts in monocyte-derived macrophages, the number of SP mRNA copies/μg total mRNA detected in preparations from four different donors ranging from 949 to 113,388 (Lai *et al*., 2002b). Therefore, given the demonstrated large variability in SP transcripts (Lai *et al*., 2002b) and depending on the number of collected AMs for the evaluation of all the other parameters (respiratory burst, cytokine release, NF-κB activation), in our AMs preparations, we evaluated NK-1R expression at the protein level, only. By using a noncommercial monoclonal anti-NK-1R antibody (raised in chicken against the final 15 amino acids at the C-terminus of the rat NK-1R), Smith *et al*. (2000) demonstrated the presence of NK-1R in human antrum and detected both a positive band at 46 kDa and a larger molecular mass band of 110 kDa, representing the glycosylated form of the NK-1R. Two NK-1R isoforms that differ in the length of the cytoplasmic carboxyl-terminus have

been reported (Fong *et al*., 1992; Mantyh *et al*., 1996; Li *et al*., 1997): in the rat, the full-length and the truncated receptor presented molecular weights of the receptor proteins of about 80 and 50 kDa, respectively, the deglycosylated receptors being 46-kDa and 37-kDa, respectively (Li *et al*., 1997). The truncated receptor, at variance from the full-length one, did not undergo rapid and long-lasting desensitization (Li *et al*., 1997); cells possessing the short NK-1R isoform would, therefore, be expected to have a prolonged responsiveness. By means of noncommercial NK-1R antibodies, a 42 kDa protein was detected in murine peritoneal macrophages and microglia (Marriott & Bost, 2000; Rasley *et al*., 2002), whereas a 53 kDa protein was demonstrated in the THP-1 cells (Simeonidis *et al*., 2003). Given the heterogeneity of NK-1R and the fact that we used a commercial polyclonal anti-NK-1R antibody, we detected three prominent bands of 68 (as indicated by the manufacturer), 53 and 42 kDa in human AMs, in accordance to what observed by others in cells of the monocyte/macrophage lineage. Interestingly, AMs from healthy smokers demonstrated a >3-fold increase in NK-1R expression as compared to healthy non-smokers: these results are in keeping with previous data indicating the key role for SP and NK-1R activation in tobacco-induced lung toxicity in both animals and humans (Dusser *et al*., 1989; Tomaki *et al*., 1995; Wu & Lee, 1999) and further extend observations from Bai *et al*. (1995), who detected an increased NK-1R expression in lung biopsies from smokers.

Furthermore, we have determined the functional nature of these NK-1R by demonstrating the ability of SP and selective NK_1 agonists to induce O_2^- production and cytokine release from human AMs. These observations extend our previous data in both human and guinea-pig AMs (Brunelleschi *et al*., 1990; 1992; 1996), demonstrating an enhanced responsiveness to tachykinins in AMs isolated from healthy smokers. By evaluating the respiratory burst, we measured a significantly increased O_2^- production when AMs from smokers were challenged with SP or selective NK_1 agonists, EC_{50}s being 0.25 nM in smokers and 1 nM in non-smokers for SP and 3 nM in smokers and 10 nM in non-smokers for $[Sar^9Met(O_2)^{11}]SP$. To further confirm the receptor nature of these effects, the non-peptide NK_1 antagonist CP 96,345 at 1 nM shifted to the right the concentration–response curve for both endogenous and synthetic NK_1 ligands.

TNF-α, IL-1β, IL-2 and IL-6 are frequently encountered proinflammatory cytokines, which are involved in a variety of immunological functions as well as interaction with different target cells. TNF-α is secreted by monocyte/macrophages mainly and, besides exerting cytotoxic activity to tumor cells, has a key role in chronic inflammation. TNF-α induces the expression of, and enhances cellular responsiveness to, other cytokines and growth factors, and affect signal transduction pathways leading to proliferation. SP and NK_1 selective agonists dose-dependently induce TNF-α release from human AMs, a more than doubled significant TNF-α release ($P<0.01$) being observed in AMs from healthy smokers. CP 96,345 at 1 nM competitively antagonize these effects, further confirming the receptor-mediated nature of SP-induced effects in human AMs. In the concentration range 1 nM–1 μM, SP also induces IL-1β release from AMs, no significant differences being observed between smokers and non-smokers. This result was somewhat unexpected also considering the fact (see below) that SP and NK-1R agonists activate the transcription factor NF-κB. As known, the regulation of TNF-α and IL-1β production is largely NF-κB-dependent, although evidence exists that TNF-α and other cytokines can also be induced through NF-κB-independent pathways (Bondeson *et al*., 1999; Andreakos *et al*., 2004). We have no definitive explanation for SP-induced IL-1β release being similar in smokers and non-smokers; however, PMA, too, although activating NF-κB and inducing cytokine release, did not release an enhanced amount of IL-1β in smokers. Moreover, AMs isolated from smokers and challenged with LPS released significantly decreased amounts of IL-1β as compared to non-smokers (Brown *et al*., 1989; Yamaguchi *et al*., 1989). Brown *et al*. (1989) concluded that 'there is a defect in release but not production of IL-1β from the AMs of chronic smokers'. In addition, SP exerts inconsistent effects on IL-10 release, in keeping with previous observations on peripheral blood mononuclear cells from healthy donors (Kim *et al*., 2003).

An important component controlling the synthesis of many cytokines and other proinflammatory gene products is the transcriptional activator NF-κB (reviewed in Baldwin, 1996). Five related mammalian gene products participate in NF-κB functions (p50/NF-κB1, p52/NF-κB2, p65/Rel A, c-Rel and RelB), the predominant species in many cell types being a p50-p65 heterodimer. As known, the transcription factor NF-κB regulates the expression of many proinflammatory genes, including those of TNF-α and IL-1β, and, in turn, these inflammatory cytokines are potent inducers of NF-κB activation (Baldwin, 1996).

SP specifically activates NF-κB in cells of the monocyte/macrophage lineage, for example, human astrocytoma cells, murine peritoneal macrophages and dendritic cells (Lieb *et al*., 1997; Marriott *et al*., 2000), but no information are available concerning human AM. We originally report here that activation of NK-1R by SP or $[Sar^9Met(O_2)^{11}]SP$ dose-dependently stimulates nuclear translocation of NF-κB, as evaluated by EMSA. This effect is reverted by the NK_1 antagonist CP 96,345. Interestingly, the entity of the effect is similar to the PMA-induced one, so indicating SP as a potent activator of this transcription factor in human AMs. Interestingly, SP induced a three-fold increase (as evaluated by densitometry) in NF-κB nuclear translocation in AMs isolated from healthy smokers as compared to non-smokers. Consistent with previous reports in which human AMs were used (Carter *et al*., 1998; Farver *et al*., 1998), a constitutive expression of NF-κB in the nucleus of unstimulated AMs was always observed in our experiments. By using specific NF-κB DNA-binding sequences for IL-6, IL-8 and TNF-α promoters, Carter *et al*. (1998) demonstrated that different NF-κB complexes are generated in AMs from healthy volunteers and that specific NF-κB complexes are used for the transcription of these cytokine genes. They found that both p50 and p65 proteins bound to the IL-6 sequence, whereas a p50 protein bound to the TNF sequence and a p65 protein bound to the IL-8 sequence (Carter *et al*., 1998).

By Western blot assays and ELISA kits, we have detected both p65 and p50 subunits in human AMs. In our experiments, the p50 subunit seems to be the most abundant one in AMs from both smokers and non-smokers, being more efficiently translocated in smokers. In fact, unstimulated AMs from healthy smokers presented a more than doubled nuclear translocation of p50 (but about the same for p65) as compared to AMs from non-smokers. When AMs were challenged by

PMA or NK-1R agonists, a further enhanced nuclear translocation of NF-κB subunits was observed: SP and [Sar^{9}Met(O$_2$)^{11}]SP were particularly effective on p50 translocation (about three-fold) and their effects were significantly enhanced in AMs from smokers. PMA, too, was very potent (more than three-fold) in inducing p50 translocation and also significantly stimulated (although to a lesser degree) p65 translocation in healthy smokers. On the contrary, SP and [Sar^{9}Met(O$_2$)^{11}]SP potentiated p65 nuclear translocation, but no significant variations were observed between smokers and non-smokers. This observation deserves further investigations but, in our opinion, is in keeping with the data demonstrating p50 as the major NF-κB subunit for the transcription of TNF-α gene (Carter *et al*., 1998). In fact, among the cytokines we evaluated, TNF-α is the one released to greater amounts by SP and NK$_1$ agonists, whereas inconsistent effect on IL-10 release are observed. As known, IL-10 exerts anti-inflammatory effects and inhibits NF-κB activation in LPS-stimulated human AMs (Raychaudhuri *et al*., 2000). Moreover, according to the fact that reactive oxygen intermediates (in particular hydrogen peroxide) have been proposed as second messenger molecules in the activation pathway of NF-κB and that antioxidants usually inhibit NF-κB activation (Lieb *et al*., 1997), the demonstrated SP ability to induce oxy-radical production could play a role in SP-induced translocation of the transcription factor. To our knowledge, this is the first paper that describes NK-1R expression and activation in human AMs as a whole, although other different reports have investigated, in monocyte/macrophages of different origin, some of the points we focused here. In conclusion, this paper indicates SP as a potent contributor for tobacco smoke toxicity.

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