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GHISLIERI
CENTRO PER LA
COMUNICAZIONE
E LA RICERCA

Progetto "Progressi in Biologia e Medicina"

15° Corso di formazione avanzata

Infiammazione, Cancro e Malattie Degenerative

17-20 maggio 2016, Collegio Ghislieri, Pavia

A cura di Giampaolo Merlini

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Prefazione

Il tema del XV Corso di formazione avanzata è il ruolo dell'infiammazione in oncologia e nelle malattie degenerative. È un argomento di grande attualità alla luce delle recenti acquisizioni sull'importante ruolo della infiammazione nella genesi e nello sviluppo del cancro e delle malattie degenerative neurologiche, cardiovascolari, e autoimmuni sistemiche. Il Corso tratta le basi biochimiche ed evoluzionistiche dell'infiammazione. Particolare attenzione sarà dedicata al ruolo dell'acido ialuronico e dei proteoglicani nella patogenesi della aterosclerosi. La genomica ha dato un contributo fondamentale alla comprensione della regolazione trascrizionale ed epigenetica dell'infiammazione. Durante il processo infiammatorio, numerose proteine della matrice extracellulare vanno incontro a degradazione enzimatica con produzione di frammenti biologicamente attivi. L'infiammazione, e la sua modulazione da parte di micro RNA, ha un impatto importante sullo sviluppo embrionale. Saranno discussi i rapporti fra infiammazione, e produzione di specie reattive dell'ossigeno (ROS), fertilità e longevità, Saranno esplorate le molteplici sfaccettature dell'immunologia del cancro e discusso come l'infiammazione associata al tumore possa essere riprogrammata con beneficio terapeutico. Saranno inoltre presentati e discussi i nuovi approcci di immunoterapia del cancro basati sulle nuove conoscenze dell'immunologia dei tumori. La flogosi cronica e l'alterata regolazione epigenetica svolgono un ruolo centrale nel processo di epatocarcinogenesi. L'infiammazione è importante anche nella patogenesi e nella evoluzione di numerose malattie degenerative sistemiche e neurologiche. Nelle malattie autoimmuni sistemiche la componente cellulare ed umorale del sistema immunitario alimentano continuamente l'infiammazione che produce danno tissutale. Questi meccanismi sono particolarmente importanti nello sviluppo del danno articolare osservato nella artrite reumatoide. Nelle amiloidosi sistemiche l'infiammazione ha un ruolo rilevante nei meccanismi di progressione e danno d'organo. L'amiloidosi reattiva è causata da una proteina di fase acuta persistentemente elevata in soggetti con malattie infiammatorie croniche. In ambito neurologico, la neuroinfiammazione ha un ruolo importante nel determinare conseguenze croniche nei pazienti che subiscono trauma cranico. Saranno discusse le basi molecolari della infettività, neurotossicità e neuroinvasività dei prioni. Il ruolo patofisiologico dei fattori dell'infiammazione e dell'immunità saranno esplorati nella malattia di Parkinson assieme alla possibilità di svilup-

pare nuovi bersagli terapeutici. La risposta infiammatoria caratterizza anche la patologia della malattia di Alzheimer ed è primariamente guidata dalle citochine e dalla microglia e promuove la neurodegenerazione. Nella sclerosi multipla le lesioni infiammatorie sono caratterizzate dalla presenza di cellule immunitarie e dall'attivazione delle cellule gliali. La diffusa infiammazione delle meningi svolge un ruolo importante nella neurodegenerazione corticale.
Le letture sono tenute da esperti internazionali e l'auspicio è che dalla diretta ed intensa interazione con i discenti, facilitata anche dal carattere residenziale del corso, possano nascere nuovi interessi e nuovi spunti di ricerca.

Giampaolo Merlini

1

Role of Hyaluronan and Proteoglycans in inflammatory responses: Implications for cardiovascular disease

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Cardiovascular diseases range among the most frequent causes of deaths worldwide and inflammatory processes have been described to be crucially involved in their pathogenesis. Atherosclerosis, a lipid-driven, chronic low-grade inflammation of the arterial wall, is the underlying cause for myocardial infarction, ischemic stroke and peripheral artery disease thereby majorly contributing to global morbidity and mortality. Atherogenic lipid retention, endothelial dysfunction and increased inflammation during atheroprogression lead to extensive remodeling of the vessel wall (1). In end stage atheroma, completely remodeled extracellular matrix (ECM), accumulation of phenotypically switched smooth muscle cells (SMC) and presence of a plethora of immune cells as well as neovascularization, necrosis of the plaque core and thinning of the fibrous cap are typically found. ECM represents a crucially involved modulator in the pathogenesis of cardiovascular diseases. Specific matrix components have been described as crucial modulators of disease progression: Biglycan (BGN), a small leucine-rich proteoglycan, is synthesized by endothelial cells, SMC, fibroblasts, and macrophages (2-4). Vascular BGN deposition occurs in the neointima and the adventitia of arterial blood vessels (5). An important aspect of the biological function of BGN is the stabilization of the fibrous cap in accordance with the known function of BGN in collagen fibrillogenesis (6). However, it was detected not only in the vessel wall, where it also contributes to vascular lipid retention (7, 8), but BGN also circulates in the plasma. This circulating BGN mediates proinflammtory functions via acting as a ligand for toll-like receptors 2 and 4 in acute highly inflammatory diseases such as sepsis and non-infectious renal injury (9, 10).
However, also anti-inflammatory actiony of BGN have been described. Using a murine model of accelerated atherosclerosis, the *Apolipoprotein E/Bgn* double-deficient mouse, we recently showed that BGN acts as an activator of heparin cofactor II via its dermatan-sulfate side-chains. In this way BGN is inhibiting direct (activation of endothelial cells and monocytes/macrophages) and indirect (activation of platelets) thrombin-mediated inflammation in atherogenesis and –progression (11). As an endogenous inhibitor of thrombin BGN may thereby indirectly affect the cardiovascular risk and the event rates.

Besides BGN, also hyaluronan (HA) exerts important disease-modulating functions. HA strongly accumulates in human primary and secondary atherosclerotic lesions (5, 12) and is thought to play a role in many aspects of atherosclerosis. The most important may be

1) to support the function of the endothelial glycocalyx (13),
2) to modulate recruitment of inflammatory cells (14) and immune cell interactions (15),
3) to modulate phenotypic switching of SMC (16-18);
4) to support neointimal expansion/luminal narrowing (19).

The endothelial glycocalyx is protective against atherosclerosis by maintaining endothelial function and by antagonizing the interaction of leukocytes and platelets with the endothelium and subsequent subendothelial inflammation and/or thrombus formation. Consequently, the inhibition of HA synthesis by 4-methylumbelliferone (4-MU) damaged the glycocalyx led to increased plaque inflammation and atheroprogression (20).

In contrast to the protective effect of endothelial HA, HA-cables and HA-fragments may contribute to the inflammatory response during atherosclerosis. In addition to that, HA is also directly involved in immune cell interactions. HA has been described as an important constituent of the so called immune synapse where it promotes dendritic cell – T cell interactions and subsequently T cell activation (15).

Furthermore, HA promotes SMC migration and proliferation and thereby may augment the establishment of a strong population of SMC within the neointima and also the expansion of the neointima driven by SMC proliferation and SMC-derived ECM accumulation. All considered it appears that HA plays a dichotomic role during atheroprogression: endothelial HA inhibits initial steps of immune cell recruitment and thereby protects against initiation and progression of atherosclerosis. On the other hand, SMC-derived neointimal synthesis of HA likely promotes atheroprogression. Accordingly, recent experiments in HA synthase (Has)3-deficient mice showed that neointimal expansion is reduced (21). Furthermore, neointimal hyperplasia and atherosclerosis was increased in HAS2 overexpressing mice (22) and neointimal hyperplasia was reduced in mice treated with 4-MU (23). In addition, the inhibition of inflammation and atherosclerosis in *Cd44/ApoE*- double deficient mice pointed towards an aggravating role of HA/CD44-signaling during atherosclerosis (23).

At the present it is not known which of the HAS isoenzymes are involved in atheroprogression largely because HAS isoenzyme-specific knock-outs have not been investigated in atherosclerosis models. The development of translational strategies to prevent the progression of atherosclerosis needs to consider the dichotomic role of HA and should focus either on the protection of the endothelial glycocalyx and/or the inhibition of interstitial HA by SMC.

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Infiammazione e sviluppo

2

Origine evolutiva, e ruolo fisiologico, della infiammazione

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Se sul ruolo fisiologico della infiammazione si trovano migliaia e migliaia di testi e piano piano le idee si vanno facendo sempre più chiare… sull'origine evolutiva di questo raffinato meccanismo di risposta adattativa alle infezioni ed ai traumi si può affermare che ancora siamo nelle condizioni di porci domande più che riuscire a dare risposte, sebbene alcune interessanti risposte già prendano forma.
Più precisamente sappiamo che l'infiammazione è alla base di una miriade di processi fisiologici e patologici e sebbene gli aspetti patologici di molti tipi di infiammazione siano ben conosciuti le loro funzioni fisiologiche sono in gran parte sconosciute.
I classici artefici della infiammazione, infezione e danni tissutali, sono alla base di una larga varietà di condizioni avverse capaci di indurre infiammazione e così facendo scatenano l'arruolamento di linfociti e proteine del plasma nel sito tissutale danneggiato. Il malfunzionamento tissutale a sua volta induce una risposta adattativa (conosciuta anche come para-infiammazione). Quest'ultima condizione dipende principalmente dai macrofagi residenti e si manifesta con uno stato intermedio tra la condizione basale di omeostasi tissutale e quella della risposta infiammatoria classica. La para-infiammazione è probabilmente responsabile delle condizioni di infiammazione cronica associate con le attuali (moderne) patologie umane.
Se questo è in breve sintesi il quadro generale che si può desumere dalla letteratura e sul quale trovare un generale accordo interpretativo (Medzhitov, 2008) è bene riassumere brevemente gli attuali pensieri sull'origine evolutiva della infiammazione, per comprenderne meglio il ruolo fisiologico. Non è qui possibile, per ragioni di tempo e di spazio, tracciare un profilo storico di queste idee a partire dalla teoria fagocitaria della infiammazione e l'intreccio con la teoria darwiniana della selezione o soffermarsi su, per quanto interessanti, profili di epistemologia genetica di questo processo.
È però necessario ed utile ricordare alcuni passaggi teorici di questa riflessione, accennando dapprima alle tipologie di molecole e cellule coinvolte nel passaggio evolutivo e poi al significato darwiniano del processo infiammatorio.

Natura della risposta infiammatoria negli invertebrati e tipi cellulari coinvolti

Può sembrare strano o ironico ai giovani studenti di medicina, mi auguro molto meno ai biologi, che uno dei padri della moderna immunologia, lo scienziato russo Elie Metchnikoff (colui che ha introdotto il termine "fagocita": "cellule circolanti in invertebrati e capaci di inglobare corpi estranei"), abbia svolto i suoi più rilevanti esperimenti in un modello animale di non-mammifero, la larva di stella di mare. Erano quelli momenti nei quali un ricercatore con ambizioni di divenire "un grande" scienziato non poteva fare a meno di frequentare la stazione zoologia Anton Dohrn (li sono iniziati gli studi di molte delle moderne discipline, dalla genetica alla fisiologia, e da li sono passati tantissimi premi Nobel). Metchnikoff impiantava spine di rosa nella larva (trasparente) di stella di mare ed era così in grado di osservare il comportamento delle cellule del sangue nell'intorno dell'impianto. Da semplici osservazioni passò poi a dimostrare l'attività fagocitaria di alcune cellule del sangue verso la comunità batterica presente nella ferita dell'impianto. Basandosi su queste osservazioni in animali invertebrati arrivò a spiegare il ruolo della fagocitosi linfocitaria nel corso della infiammazione nell'uomo. Anche gli allievi di cotanto maestro impiegarono modelli animali di invertebrati e così era giunta l'ora di Drosophila e degli insetti (principalmente di quelli di interesse economico o perchè nocivi o utili nella lotta biologica) ed infatti uno degli allievi di Metchnikoff, Serge (Serguei) Metalnikov studio intensamente i meccanismi della fagocitosi negli insetti. Per più di trent'anni si dedicò allo studio dei vari tipi di emociti degli insetti scoprendo che gli emociti granulari (granulociti) costituiscono la principale componente fagocitaria dei batteri a livello dei noduli infettivi nelle larve di lepidotteri (*Galleria mellonella* ((la tarma della cera delle api)) e *Pyrausta nubilirasia* ((il naturale competitore del *verme* del mais)). Da questi lavori "storici" appare chiaro che anche in animali erroneamente considerati "inferiori" è presente quella costellazione di reazioni e meccanismi cellulari/molecolari che chiamiamo infiammazione e che nulla ha di meno complesso di quella che vediamo in azione nei mammiferi (Metalnikov, 1924). Va comunque precisato che la mancanza di linfociti e immunoglobuline fa del sistema immunitario degli invertebrati un sistema ben più dipendente da difese non-specifiche quali la infiammazione al fine di mantenere l'integrità fisiologica. Risulta quindi di estremo interesse vedere alcuni dettagli della risposta infiammatoria negli invertebrati e nei "bassi" vertebrati, analizzando in particolare la natura delle cellule coinvolte ed i mediatori chimici delle risposte cellulari, al fine di meglio comprendere quella articolata sinfonia di reazioni che costituisce la infiammazione nei mammiferi. La fagocitosi è un fenomeno universale ad ogni livello di organizzazione del mondo animale: meccanismo di assunzione di risorse trofiche negli unicellulari, evolve come meccanismo di difesa utile a mantenere l'integrità del corpo degli animali pluricellulari. Diversi sono i tipi cellulari preposti alla fagocitosi e la letteratura scientifica è ricca di termini al riguardo (vedi oltre). Molti degli invertebrati sono dotati di un ampio celoma ripieno di liquidi e cellule e così queste ultime sono dette celomociti è un termine assai diffuso per gli invertebrati; per quegli invertebrati nei quali il celoma è assai ridotto e la cavità corporea più ampia è l'emocele (insetti,

molluschi ed urocordati ad esempio) il termine di riferimento per le cellule presenti è emociti. E così, negli animali ove è presente un celoma ed un emocele egualmente ben rappresentati le cellule vengono indicate come celomociti ed emociti (ad esempio negli anellidi). Poiché tutti gli organi sono bagnati o dal liquido celomatico o dal sangue risulta evidente che in tutti gli animali sino ad ora menzionati è possibile un rapido dislocarsi di celomociti o emociti nel sito di danno tissutale o di invasione batterica rendendo altamente efficiente la risposta infiammatoria. Diversi sono i termini impiegati per riferirsi a questi tipi cellulari: così il fagocita è chiamato amebocita, immunocita, macrofago, cellula ialina, granulocita, plasmocita, cellula granulare. L'impiego di termini presi ad uso dalla ematologia dei mammiferi non implica assolutamente alcuna relazione evolutiva o filogenetica. Non è ancora chiaro se le cellule fagocitarie del sangue degli invertebrati abbiano originato granulociti e macrofagi o entrambi i tipi cellulari dei vertebrati; d'altro canto è chiaro che i macrofagi sono un tipo cellulare più antico rispetto ai granulociti. È interessante notare che mentre la gran parte degli invertebrati possiede un solo tipo di linfociti fagocitari, qui e la vi sono degli animali ove sia i granulociti basofili sia quelli eosinofili sono dotati di attività fagocitaria (ad esempio nel comune mollusco bivalve *Mytilus edulis*); un altro esempio è quello di *Ciona intestinalis* (urocordato) ove sia gli amebociti ialini sia quelli granulari sono attivamente fagocitari. Non è chiaro se questi tipi cellulari facciano parte di una unica serie maturativa, come nel caso dei monociti e macrofagi dei mammiferi oppure appartengano a distinte linee cellulari, come nel caso dei granulociti e dei macrofagi sempre dei mammiferi.

Due sono i principali processi caratteristici della risposta infiammatoria negli invertebrati: fagocitosi e incapsulamento, il primo molto simile al processo di fagocitosi nei mammiferi (se non per la differenza nel processo di riconoscimento cellulare), il secondo presenta solo alcune somiglianze alla formazione dei granulomi nei mammiferi. Il meccanismo di eliminazione intracellulare del materiale fagocitato implica il coinvolgimento di diversi tipi di molecole quali radicali liberi, generazione di ossido nitrico (monossido di azoto, NO) e svariati enzimi idrolitici (inclusi lisoenzimi). L'incapsulamento si verifica nel corso della cicatrizzazione tissutale, di una invasione parassitaria o batterica; in quest'ultimo caso ci si riferisce alla formazione di noduli o nodulazione. Negli artropodi il processo è stato studiato a lungo con la scoperta che si tratta di un fenomeno bifasico: il primo evento è la degranulazione di cellule instabili capaci di rilasciare fattori pro-infiammatori; il secondo implica il completo avvolgimento (la completa ricopertura) di queste cellule da parte dei fagociti con la efficace eliminazione dei parassiti o degli agenti microbici. Nel corso del riparo tissutale il primo evento è utile per "tappare" la ferita così da limitare la perdita di sangue mentre la successiva ricopertura rafforza questa prima risposta e fornisce il retroterra citoarchitetturale per la rigenerazione dei tessuti.

I mediatori della risposta infiammatoria negli invertebrati possono essere suddivisi in alcune famiglie di molecole:

- citochine simili: sia tra i protosmoni sia tra i deuterostomi è evidente la presenza di molecole citochino-simili ad attività pro-infiammatoria. Il primo dato significativo a questo riguardo è riportato ben quarant'anni orsono da Prender-

gast e Liu (1976) i quali trovano nel sangue della stella marina *Asterias forbesi* una citochina di 38kDa capace di stimolare la chemotassi monocitaria e la attivazione macrofagica nei mammiferi. Ormai trent'anni orsono Beck e Habicht (1986) trovarono, sempre nella stella marina, una proteina di 29.5kDa simile alla IL-1 la cui attività biologica può essere inibita dall'anticorpo policlonale contro la IL-1 di mammifero con ciò dimostrando una certa conservazione evolutiva della sequenza di questa molecola. Nell'insieme vi sono diverse evidenze a favore dell'idea che negli invertebrate esistano citochine equivalent a quelle dei mamiferi.

- il sistema di attività profenolossidasi: la melanizzazione che si può mettere in evidenza nei noduli e nelle capsule emocitarie degli artropodi (in particolare negli insetti e nei crostacei) come risposta ad agenti infettivi è un fenomeno ampiamente diffuso. Più di trent'anni orsono questa associazione tra melanina e infiammazione era già stata individuata come un meccanismo di eliminazione basato sulla formazione intermnedia di chinoni tossici nel corso della generazione di melanina. Un ruolo central in questa cascata di reazioni è giocato dall'enzima fenolossidasi che si trova all'interno degli emociti o nel plasma sotto forma di proenzima (profenolossidasi: questo sistema è attivato da prodotti del metabolism microbico capaci di indurre il taglio della profenolossidasi grazie ad attività proteinserasiche.
- Eicosanoidi: è noto che gli eicosanoidi (in particolare il leucotriene B4, LT B4) giocano un ruolo centrale nella infiammazione dei mammiferi e dunque non appare strano il fatto che molti studiosi abbiano tentato di mettere in evidenza il potenziale ruolo di mediatori della infiammazione di queste molecule anche negli invertebrati: e però, con la eccezione del veleno degli artropodi, non è stato possibile avere riscontri sulla presenza di attività 5-lipossigenasi e LTA idrolasi, attività necessarie per la generazione di LT B4.
- molecole di adesione: le molecole di adesione superficiale della famiglia delle integrine sono note avere una lunga storia evolutiva essendo state ben caratterizzate in Drosophila e nei pesci.

Sulla scorta della presenza di queste tipologie di molecule è possibile analizzare la natura della risposta infiammatoria nei vertebrati "inferiori" ed i tipi cellulari coinvolti. È bene ricordare che lo stadio più significativo nel corso della evoluzione del sistema immunitario è quello concomitante alla comparsa dei primi vertebrati: probabilmente questi sono stati I primi animali ad essere dotati di un "vero" sistema immunitario con "veri" linfociti dotati della abilità di rispondere grazie ad una selezione clonale ad agenti microbici. Inoltre è molto probabile che questi animali fossero anche dotati dlla abilità di sintetizzare "vere" immunoglobuline (in alter parole molecule con regioni strutturali variabili) capaci di interagire con molecole non-self ed anche con molecole alterate del self. Gli attuali antenati di questi primi vertebrati sono i pesci. Poiché i vertebrati più primitivi erano privi di mandibola (agnati) si ritiene che pesci quali le lamprede siano gli attuali rappresentanti di queste prime forme (gli altri pesci, gnatostomi (dotati di mandibola) si dividono in pesci cartilaginei, razze e squali, e pesci ossei, carpa, trota, etc.). E dunque i pesci sono un gruppo di estremo interesse per esaminare l'evoluzione dei

sistemi infiammatori. Gran parte degli studi a questo riguardo sono stati rivolti a caratterizzare citochimicamente e molecolarmente le propietà dei tipi di leucociti coinvolti nella infiammazione (Rowley, 1996; Medzhitov, 2008; Okin e Medzhitov, 2012). È così emerso che non solo i linfociti hanno fatto la loro comparsa nel corso della evoluzione dei vertebrati ma anche gli altri tipi di leucociti (i.e., granulociti e monocytes/macrophages) si sono evoluti durante queste fasi con la sola possibile eccezione delle mast cellule (cellule per altro chiaramente identificate in tutti gli altri tipi di vertebrati. Certamente i pesci sono un'area di studio e controversie per quanto riguarda i tipi cellulari del sangue e così non stupisce che anche per i granulociti vi sia ancora dibattito in particolare per quanto riguarda la eterogeneità funzionale dei granulociti.

Infiammazione nella prospettiva evolutiva

Alcune riflessioni si impongono sotto il profilo evolutivo per quanto riguarda il significato funzionale della infiammazione ed in particolare è necessario riflettere sui fattori, e quali, che hanno contribuito alla evoluzione della infiammazione quale meccanismo di difesa ed il loro ruolo nell'attuale incidenza (aumentata) delle malattie a base infiammatoria. È cioè bene riflettere sulla natura duale di alcuni di questi fattori e del ruolo giocato dalle forze evolutive nel loro mantenimento.
Per prima cosa è bene sottolineare che il guadagno nel rapporto costi-benefici delle difese immunitarie è stato ottimizzato (dalla evoluzione) per un ambiente che chiaramente non esiste più, certamente non più per le popolazioni delle società occidentali e dei paesi maggiormente industrializzati. E in seconda battuta chiedersi perchè mai la risposta infiammatoria è sempre associate con condizioni patologiche; per capire appieno questo aspetto è bene ricordare seppur concisamente alcuni principi di evoluzione dei tratti adattativi (argomento quanto mai desueto nei programmi di studio non solo dei medici ma addirittura dei biologi!). E ricordare che non sempre l'evoluzione produce soluzioni ottimali e che a volte (in precise condizioni) alcuni tratti possono evolvere senza avere alcun valore adattativo. Basterà ricordare alcune semplici nozioni. La prima è che una particolare caratteristica può essere adattativa se è stata selezionata a favore perchè aveva un effetto positivo sulla fitness dell'organismo (in ultima analisi, ne beneficiava il successo riproduttivo). È importante ricordare che simili tratti sono adattativi nelle condizioni ambientali presenti esattamente quando questi tratti evolvettero. Se quelle condizioni ambientali cambiano quelle stesse caratteristiche possono essere maladattative (obesità e allergie sono classici esempi di tratti maladattativi). La seconda è che un certo tratto può essere non-adattativo quando esiste quale conseguenza di un altro tratto (adattativo), anche se di per sè non ha alcun valore adattativo: un esempio di questa seconda condizione è lo shock anaffilattico e la distruzione tissutale da parte dei neutrofili attivati. Questi tratti non-adattativi non sono stati selezionati a favore, dimostrandosi neutri o detrimentali per la fitness di un organism (e nelle condizioni in cui si sono evoluti) ma esistono come conseguenza inevitabile di altri tratti adattativi. Questa seconda condizione è più comune di quanto si creda e costituisce

un bilancio (guadagno) evolutivo tra gli effetti beneficiali di tratti adattativi ed effetti detrimentali di tratti non-adattativi con i quali sono associati. Ciò che va ricordato è che un cambio nelle condizioni ambientali può far scivolare questo bilancio da non-adattativo a detrimentale. Questa seconda condizione si presenta più frequentemente nel corso evolutivo per le specie di recente comparsa, come nel caso dell'uomo che non ha ancora raggiunto un punto di equilibrio evolutivo. Molte delle attuali moderne patologie sono il risultato di guadagni evolutivi sbilanciati causati da drastici cambiamenti delle condizioni ambientali e degli stili di vita. E così, quando si considerino i processi infiammatori vi sono molti esempi di tratti adattativi e non-adattativi in gioco. Così per molti dei processi infiammatori cronici balzano evidenti gli aspetti patologici (maladattativi) mentre non vi è immediate riscontro dei loro effetti fisiologici (adattativi) che si presume esistano. Distinguere tra caratteristiche adattative e non-adattative è essenziale non solo per capire a fondo il significato funzionale della infiammazione ma anche per sviluppare strategie terapeutiche efficaci. Ad esempio, sia i processi adattativi sia quelli non-adattativi possono contribuire alla sintomatologia di una specifica patologia ma l'interferire solo con i processi adattativi può peggiorare la sintomatologia mentre il bloccare quelli non-adattativi può risultare di beneficio senza causare effetti avversi.

È evidente che il guadagno di processi di costi-benefici evolutivi delle difese infiammatorie è stato ottimizzato per un ambiente che non esiste più per la gran parte della popolazione umana. Inoltre, l'adattamento ad un ambiente può attuarsi attraverso diversi meccanismi tra I quali l'adattamento genetico grazie alla selezione natural capace di far variare la frequenza allelica di tratti "positive" o anche tramite meccanismi di adattamento fisiologico non genetici. Ancora, molti animali sono in grado di alterare l'ambiente al fine di soddisfare esigenze specifiche, processo conosciuto come "costruzione di nicchia"; esempi di costruzione di nicchia umana sono lo sviluppo di pratiche di agricoltura, le diete, l'urbanizzazione, l'uso di vestiario e dei prodotti per la igiene personale, medicine e antibiotici. Questo rapido (in termini evolutivi) cambiamento della nicchia e di molti fattori ambientali comporta alterazioni della bilancia di costi-benefici per molti tratti, compresa la risposta infiammatoria che può divenire causa di alterate condizioni fisiologiche e promotrice di patologie quali malattie atopiche, cardiovascolari, obesità e diabete di tipo 2. Queste riflessioni valgono in assoluto ancora di più nel caso della infiammazione rispetto ad altri processi poichè l'infiammazione è un processo intrinsecamente di alto beneficio, alto costo, e dunque altamente vulnerabile anche da minimi cambiamenti ambientali. Così anche solo una condizione di sub-ottimale bilancia di costi-benefici può avere un altissimo impatto negativo sulla fitness e causare patologie; ciò è particolarmente vero per le infiammazioni di tipo cronico dell'età avanzata. L'attivazione dei meccanismi di controllo della infiammazione che porta alla infiammazione cronica altera ancora di più lo stato funzionale e viene a creare un circolo vizioso che determina le comuni patologie infiammatorie metaboliche e neurodegenerative, ad esempio: vale la pena di sottolineare che in questo contesto le comuni malattie infiammatorie sono da ritenersi malattie di alterata omeostasi.

Da ultimo vale la pena ricordare che l'infiammazione può promuovere diverse malattie alterando specifici tratti della storia del ciclo vitale. L'infiammazione è indotta da stimuli ambientali nocivi e quindi può essere segnale di un ambiente sfavorevole.
In conclusione, la prospettiva evolutiva è capace di fornire suggerimenti sulla suscettibilità ai processi infiammatori così come chiarire alcuni aspetti dell'aumentata incidenza di specifiche malattie nelle attuali popolazioni umane, sottolineando ironicamente come una ridotta mortalità da infezioni, fame, malnutrizione, predazione (per citare alcune fonti di selezione) ha creato uno scenario favorevole per la vulnerabilità a nuove patologie.

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3

Infiammazione, microRNA e sviluppo embrionale

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È stato dimostrato che l'attivazione della risposta infiammatoria anche al di fuori del tratto genitale (ad esempio nel corso di una mastite) è in grado di determinare perdite embrionali (Hansen et al. 2004) con ciò causando disordini riproduttivi e riducendo il tasso di fertilità (per una review si veda Weiss et al., 2009). Ciò che non è chiaro è se l'infiammazione (esterna al tratto genitale) può danneggiare l'embrione anche nella fase preimpianto del suo sviluppo. Le fasi precoci dello sviluppo embrionale sono infatti molto sensibili alla qualità dell'ambiente nel quale l'embrione si sviluppa (per una review si veda Fleming et al., 2004 e Fabian et al., 2005). Gli studi di cui si dispone hanno già sostenuto *in vitro* questa realtà dimostrando che la presenza nelle soluzioni di coltura cellulare di vari mediatori della infiammazione è in grado di alterare significativamente le capacità di sviluppo embrionale. (Wuu et al., 1999; Bedaiwy et al., 2005; Kawamura et al., 2007). Solo recentemente è stato però possibile condurre esperimenti *in vivo* al fine di verificare la loro dannosità: impiegando un modello animale murino è stata studiata la qualità dello sviluppo embrionale in condizioni di due diversi tipi di infiammazione acuta, entrambi localizzati al di fuori del tratto genitale. Entrambe le infezioni sono state scelte perché piuttosto comuni e frequenti: l'una riconducibile ad una forma di colite e l'altra ad una infezione generalizzata del piede (infezione indotta da carragenina, un test di infiammazione acuta, non immune, sperimentalmente ben studiato ed altamente riproducibile). Per la prima sono stati impiegati trattamenti con acido trinitrobenzen-sulfonico (TNBS) capace di indurre una forma di colite acuta mimica della infiammazione indotta da apteni e che esibisce delle caratteristiche comparabili ai processi autoimmunitari dovuti alle cellule T-helper di tipo I. La somministrazione rettale di TNBS causa una rottura (focale) della barriera epiteliale ed attiva le cellule immunitarie dell'intestino. L'infiammazione è caratterizzata dalla sovraespressione, a livello della mucosa, del fattore di necrosi (TNF), interferon ed interleukina-1 (IL-1) che porta al reclutamento di granulociti neutrofili ed alla produzione di proteasi e di fattori pro-apoptotici (Vergnolle et al., 2004; Li et al., 2008). Questo processo è anche accompagnato dall'attivazione di diversi enzimi (COX-2, iNOS) e dall'aumentata espressione di altre citochine pro-infiammatorie (interleukin-17, interleu- kin-18; Maerten et al., 2004; Zhang et al., 2007) o fattori di crescita profibrinogeni (transforming growth

factor 1, insulin-like growth factor 1) capaci di stimolare la proliferazione di cellule mesenchimali (Lawrance et al., 2003). Per la seconda è stato studiato l'edema indotto da carragenina, il comune saggio per valutare l'azione pro-infiammatoria o anti-infiammatoria di diverse molecole. In un primo momento infatti l'iniezione nel piede posteriore di un roditore causa una locale sovra produzione di istamina, serotonina e 5-idrossitriptamina e del fattore di attivazione piastrinica capaci di mediare la vasodilatazione, l'extravasazione del plasma e la secrezione di diverse citochine (TNF-α, IL-1x, IL-6); l'essudazione diviene massima tra 4 e 6 ore dopo l'induzione dell'edema. La seconda fase dell'infiammazione è caratterizzata dal rilascio di bradichina, prostaglandine e dall'azione di neutrofili polimorfi. Come è ben noto, la migrazione linfocitaria è accompagnata dalla produzione di radicali liberi di ossigeno, idrolitici e capaci di indurre la perossidazione dei lipidi e danno cellulare (Fantone et al., 1982).

Entrambe le condizioni sperimentali impiegate sono note per indurre una forte infiammazione a livello locale, di tipo focale, sebbene come in altri processi di infiammazione acuta siano accompagnati anche da risposte sistemiche. Si presentano dunque come condizioni sperimentali capaci di dare indicazioni sulle capacità di sviluppo embrionale e sulla qualità degli embrioni al termine della fase pre-impianto, nel corso dello sviluppo fetale e dei neonati. È da notare che grazie a questo tipo di condizioni sperimentali è possibile stabilire con precisione il momento dello sviluppo embrionale nel quale far agire le condizioni di infiammazione sullo sviluppo: nelle condizioni ora indicate è stato scelto di accendere l'infiammazione al momento della divisione che porta alla formazione della morula così da poter seguire le eventuali conseguenze fisiologiche sullo sviluppo embrionale *in vivo* sino allo stadio di blastocisti. A questo livello gli embrioni sono stati isolati e coltivati *in vitro* per altre 24 ore. Al termine della sperimentazione sono stati valutati dei parametri basilari dello sviluppo embrionale:

a) crescita embrionale;
b) incidenza di morte cellulare.

Dati sulle risposte sistemiche associate con i due modelli sperimentali impiegati da Bystriansky e colleghi (2010) suggeriscono che nel corso dello sviluppo dei processi infiammatori nel corpo materno la composizione del microambiente ove l'embrione va sviluppandosi possa cambiare sia in modo diretto (trasferimento di mediatori della infiammazione dal siero al fluido dell'ovidotto) sia indiretto (come risultato della sensibilità alle citochine da parte del sistema ovidotti-utero). L'insieme dei dati ottenuti (Bystriansky et al., 2010) suggerisce che le infiammazioni non specifiche localizzate al di fuori del tratto genitale non determinino effetti evidenti sulla crescita degli embrioni nella fase preimpianto del loro sviluppo. Comunque, le blastocisti derivate da morule influenzate *in vivo* dalla presenza di processi infiammatori dimostrano chiaramente un significativo aumento di processi apoptotici e di morte cellulare. La relativa bassa sensibilità della crescita embrionale alle condizioni di infiammazione può essere dovuta alla bassa concentrazione dei recettori espressi dall'embrione nei confronti dei mediatori della infiammazione, espressione che raggiunge un suo picco fisiologico di massimo al termine del periodo di sviluppo pre-impianto. E comunque resta il fatto che

aumenta la morte cellulare per apoptosi in tutti gli embrioni pre-impianto che hanno subito l'influenza di mediatori della infiammazione. Con estrema cautela si potrebbe suggerire che questo ultimo dato potrebbe essere il risultato non tanto dell'esposizione *in vivo* alle condizioni di infiammazione quanto piuttosto alle mutate condizioni della coltura *in vitro* nelle successive 24 ore di coltura. È noto infatti che le condizioni di coltura cellulare sono in grado di costituire dei fattori di stress capaci di potenziare le manifestazioni di processi "negativi" di crescita embrionale (Pomar et al., 2005). Sotto questo profilo è quindi possibile sostenere con cautela che se gli embrioni si fossero sviluppati ancora in vivo la situazione avrebbe potuto essere ben diversa; in altre parole le differenze registrate di qualità embrionale (valutata come maggior incidenza di morti cellulari e non di alterata crescita embrionale) avrebbero potuto essere non significative. Tuttavia questa cautela non deve essere posta a salvaguardia di condizioni di infiammazione ininfluenti sullo sviluppo embrionale poiché la maggiore incidenza di morte cellulare è lì a dimostrare l'esistenza di cambiamenti nella fisiologia cellulare indotti dalle condizioni di infiammazione. E dunque questa condizione è certamente meritevole di più approfondite indagini. Sono questi i dati più recenti di cui disponiamo e parrebbe di estremo interesse tornare ad indagare lo sviluppo embrionale in condizioni di infiammazione sistemica, anche impiegando nuovi strumenti di analisi delle attività genomiche quali quelli che l'analisi dei microRNA è in grado di assicurare (Pan e Chegini, 2008) nello studio del microambiente tubarico. Lo studio dei miRNA è divenuto infatti un potente strumento di indagine molecolare di svariati processi fisiologici, inclusa la infiammazione, nel corso della gravidanza (Hailemariam et al., 2013) poichè assicura di evidenziare sottili modificazioni dell'espressione genica così come già dimostrato per la attivazione differenziale di miR-146 and miR-155 in condizione di sensibilizzazione immunitaria innata (Schulte et al., 2013).

Ancor più recentemente sono apparse in letteratura evidenze che suggeriscono una maggior cautela nell'affermare la (sebbene non sostanziale) ininfluenza dei processi infiammatori al di fuori del tratto genitale sullo sviluppo preimpianto dell'embrione. Nonostante la mancanza di una diretta connessione tra il corpo materno e l'embrione in precoci fasi di sviluppo, l'embrione è in grado di comunicare con l'ambiente materno (e viceversa) e di rispondere a disordini dell'omeostasi di tale relazione. Le citochine espresse dall'embrione pre-impianto, ad esempio, sono in grado di giocare un ruolo di primo piano nel processo di impianto modificando l'attività proliferativa delle cellule degli ovidotti o le secrezioni dell'ovidotto stesso (Herath et al., 2009). La più recente letteratura presenta infatti una serie di dati che suggeriscono l'esistenza di una tale influenza, sostenendo che alterazioni nell'espressione di mediatori degli stati proinfiammatori possano essere responsabili di mancato successo dell'impianto embrionale uterino e di diversi tipi di disordini riproduttivi (Jaiswal et al., 2006; Chapwanya et al., 2009).

Sta facendosi sempre più chiaro a questo proposito il ruolo svolto da svariati micro-RNA: questi giocherebbero un ruolo strumentale nell'arena dei processi infiammatori in relazione allo sviluppo embrionale, come recentemente dimo-

strato da Ibrahim et al. (2015) in un modello sperimentale bovino. In questo studio gli AA analizzano la sopravvivenza degli embrioni in condizioni di infezione batterica delle cellule dell'ovidotto dimostrando che numerosi miRNA, con la sola eccezione di miR-21, sono sovraespressi. Nel loro insieme questi dati evidenziano l'instaurarsi di un ambiente sub-ottimale negli ovidotti capace di alterare le capacità dell'embrione di impiantarsi con successo. Di particolare rilievo nello stabilirsi di queste condizioni sub-ottimali sarebbe l'instaurarsi di fenomeni apoptotici nelle cellule epiteliali degli ovidotti. È noto che l'apoptosi è associata con la qualità e la capacità di impianto negli stadi di sviluppo embrionale pre-impianto: l'apoptosi ha una maggior incidenza nel caso di condizioni di sviluppo sub-ottimali (Makarevich et al., 2008). Nel caso di stimolazione delle cellule dell'ovidotto con lipopolisaccaridi (Tili et al., 2007) si attivano diversi segnali di trasduzione del segnale capaci di evocare la formazione di una pletora di mediatori biochimici, incluse citochine (TNF-α *e CSF1), radicali liberi (tossici, chiaramente) e di modulare l'*espressione di miR-155 e miR-125b evidenziandone il ruolo nella risposta endotossica sull'epitelio degli ovidotti. È evidente che una gravidanza per avere successo richiede una delicata bilancia tra molecole pro-infiammatorie (Th1) e anti-infiammatorie (Th2) per mantenere una sostanziale integrità del sistema immunitario (Walker et al., 2010; Kowsar et al., 2013) così da prevenire il rigetto dell'embrione (mancato impianto). Ne consegue che il disturbo nella qualità e quantità di mediatori della infiammazione è in grado di esercitare un effetto inibitorio sulla crescita (delle cellule e dell'embrione) ed un'aumentata incidenza dell'apoptosi. È evidente che ogni modificazioni di questa bilancia tra fattori pro- e anti-infiammatori finemente regolata dai micro RNA è in grado di determinare il successo dell'impianto dell'embrione: ne consegue la attenzione che deve essere posta alla influenza dell'ambiente inteso in senso lato allo stabilirsi di condizioni di infiammazione nel corpo materno nel corso delle primissime fasi dello sviluppo embrionale.

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4

Infiammazione, fertilità, longevità

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Infiammazione e longevità

Invecchiando ci sono più probabilità di diventare fragili, meno attivi e di sviluppare malattie cardiache, diabete e altre malattie legate all'invecchiamento. Questo, in parte, è dovuto a danni a tessuti e organi che si accumulano nel corso della nostra vita e la capacità di invecchiare bene o male è indubbiamente legata al nostro DNA e alle interazioni con l'ambiente in cui viviamo. Danni al DNA, proteine e altre molecole essenziali per il nostro organismo sono causati dai radicali liberi e, in modo particolare dalle specie reattive dell'ossigeno (ROS), ovvero i radicali liberi a maggior diffusione, che svolgono un ruolo cruciale nell'influenzare il modo in cui invecchiamo. ROS vengono prodotti durante la respirazione, le risposte immunitarie e altri importanti processi cellulari ma, se prodotti in quantità eccessive, possono essere estremamente dannosi. I livelli di ROS vengono controllati, a livello cellulare, in diversi modi, proprio per evitare danni al DNA e altre molecole importanti.
Una delle altre caratteristiche dell'invecchiamento è l'insorgenza di una infiammazione cronica in diversi tessuti che è parzialmente attivata dal sistema immunitario in risposta al danno cellulare. Esiste un gruppo di geni chiamati CD33rSIGLEC coinvolti nel controllo dell'infiammazione. Uno dei motivi per cui alcuni tipi di animali (i.e. mammiferi) vivono più a lungo rispetto ad altri, potrebbe essere dipendente dal numero e dall'attivazione proprio di questi geni. Infatti, le specie che hanno più copie di CD33rSIGLEC hanno una durata di vita più lunga. È stato dimostrato che topi che presentano un minor numero di copie di questi geni, infatti, invecchiano precocemente e muoiono prematuramente rispetto a topi di controllo e presentano livelli più elevati di ROS, comportando questo una maggiore quantità di danno al DNA (Angata et al., 2004; Schwarz et al., 2015). Le SIGLEC sono una famiglia di lectine animali che appartengono alla superfamiglia delle Ig leganti acido sialico e le CD33rSIGLEC ne costituiscono un sottogruppo la cui funzione è proprio quella di intervenire contro il processo infiammatorio.
Nel corso degli anni è stato possibile dimostrare come CD33rSIGLEC sia in grado di controllare i livelli di ROS durante l'infiammazione, riducendo così i danni alle cellule ed estendendo la durata della vita degli animali (Schwarz et al., 2015).

CD33rSIGLEC

Si conoscono due classi di SIGLEC, definite sulla base di omologia di sequenza e conservazione. Il primo gruppo (Sialoadhesin/Siglec-1, CD22/Siglec-2, MAG/Siglec-4 e Siglec-15) condivide una bassa identità di sequenza ed è altamente conservato tra i mammiferi. Al contrario, i geni codificanti per CD33rSIGLEC vanno incontro ad estesi riarrangiamenti, tra cui duplicazione, conversione, pseudogenizzazione e quindi variano in numero e sequenza tra diverse specie di mammiferi.
Ad esempio, topo e uomo esprimono cinque e undici CD33rSIGLEC funzionali definiti da una nomenclatura alfabetica e numerica rispettivamente (nell'uomo sono SIGLEC -3, -5, -6, -7, -8, -9, -10, -11, -XII, -14 e -16). Sebbene informazioni riguardanti modelli di espressione di SIGLEC non siano complete, è noto che siano però cellula-specifici. Per esempio, tra le CD33rSiglecs murine, CD33 è espressa prevalentemente in granulociti, Siglec-E è espressa principalmente in neutrofili, monociti, microglia e cellule dendritiche, Siglec- F si trova principalmente in eosinofili e mastociti, Siglec - G è prevalentemente espressa in cellule B e alcune cellule dendritiche mentre Siglec-H è espresso principalmente in cellule dendritiche plasmacitoidi (Pillai et al., 2012). Sebbene non sia possibile individuare ortologhi CD33rSIGLEC umani e murini, a causa della rapida evoluzione di SIGLEC e la profonda divergenza temporale, alcuni recettori (ad esempio, Siglec-E e Siglec-9) sono considerati omologhi funzionali (Läubli et al., 2014).
CD33rSiglecs sono in grado di trasmettere segnali inibitori in cellule immuni dalla fosforilazione di ITIM intracellulare (cytoplasmic immunoreceptor tyrosine-based inhibitory motifs) o domini ITIM-like, spegnendo l'attivazione di eventi pro-infiammatori.

Tab. 1 - Correlazione tra longevità, massa corporea ed espressione di diversi tipi di geni, tra cui quelli appartenenti alla famiglia delle SIGLECs.

Species	Maximum lifespan (years)	Average adult body weight (g)	Number of genes			
			SIGLECs	FeRs	TLRs	KLKs
Mus Musculus	4	21	5	4	12	26
Monodelphis domestica	5	105	4	2	10	8
Canis familiaris	24	40000	6	3	9	13
Callithrix jaccus	16,5	255	5	4	9	12
Bos taurus	20	750000	6	4	10	13
Macaca mulatta	40	8235	8	4	11	11
Pongo pygmaeus	59	64475	8	4	10	14
Homo sapiens	120	62035	10	8	9	15
Pan troglodytes	59	44984	9	5	10	11
Loxodonta africana	65	4800000	10	4	10	7
Equus caballus	57	250000	10	4	10	13
Felis catus	30	3900	5	2	12	6
Sus scrofa	27	180000	6	2	11	0
Rattus norvegicus	4	300	4	6	12	22

Recentemente, è stato dimostrato che Siglecs può controllare direttamente il recettore Toll-like (TLR) di segnalazione per sostenere le interazioni acido sialico-dipendente con TLR e CD14 (Chen et al., 2014; Ishida et al., 2014). Sempre recentemente è stato dimostrato che il numero di geni CD33rSiglecs è correlabile con la durata di vita dei mammiferi.
In particolare, lo spegnimento di Siglec-E influisce su la longevità dei topi. Infatti, topi Siglec-E deficienti mostrano segnali di inve chiamento accelerati rispetto ai controlli con un aumento del tasso di danno ossidativo a livello sistemico ed una riduzione della longevità.
CD33rSiglecs regola dunque il danno infiammatorio e, come è possibile notare dalla tabella 1, più geni appartenenti a questa famiglia delle SIGLECS sono espressi, più lunga è l'aspettativa di vita in diversi tipi di mammiferi (Swarz et al., 2011).

Infiammazione e fertilità

Ad oggi, molte sono le conoscenze che correlano il processo infiammatorio con la fertilità femminile e, in modo particolare, con l'insorgenza di determinate patologie come nel caso dell'endometriosi.
L'endometriosi è definita dalla presenza di tessuto endometriale (stroma e ghiandole) fuori dalla cavità uterina. La prevalenza stimata di endometriosi in donne asintomatiche è 2-20%, a seconda dei criteri diagnostici. Nelle donne con dolore pelvico, la prevalenza varia da 15 a 45%; si stima che il 30-70% delle donne infertili soffrono di endometriosi e che tra le donne con endometriosi il 30-50% sono infertili (Mahmood e Templeton, 1991). L'endometriosi può influenzare diverse fasi del ciclo riproduttivo femminile, a partire dal processo di follicologenesi, di disfunzione ovulatoria, di ridotta steroidogenesi preovulatoria di cellule della granulosa, di fecondazione alterata e di difetti nelle fasi di impianto dell'embrione, solo per citarne alcuni (Halis e Arici, 2004). Ad oggi, vi sono ancora molti punti oscuri legati alle cause che portano a sviluppare endometriosi ed infertilità e molti di essi sono dovuti alla qualità degli studi sino ad ora avviati, condotti principalmente su un numero non significativo di pazienti arruolati e senza tenere in considerazione gruppi di controllo. Tuttavia, recenti analisi hanno dimostrato che il liquido peritoneale delle donne con endometriosi contiene quantità elevate di macrofagi, fattori di crescita, citochine, e fattori angiogenetici, tutti mediatori pro-infiammatori che interesserebbero vari aspetti della riproduzione femminile.
Le cellule del sistema immunitario svolgono un importante ruolo modulatore tra i diversi tipi cellulari che costituiscono l'ovario. Nel liquido follicolare di donne con endometriosi, il livello di VEGF (vascular endothelial growth factor) risulta essere diminuito mentre i livelli di interleuchina-1 (IL-1), IL-6, IL-8, fattore di necrosi tumorale-α (TNF-α), diverse cellule natural killer, linfociti B e monociti (MCP-1), endotelina-1, risultano essere elevati. Alti livelli di TNF-α sono stati inoltre correlati con una scarsa qualità delle cellule uovo e insieme ad alti livelli di IL-1 hanno la funzione di inibire la produzione di progesterone da parte delle cellule della granulosa e di androgeni dalle cellule della teca.

Analizzando le cellule della granulosa di pazienti con endometriosi e, in particolare studiando l'incidenza di apoptosi, cambiamenti nel ciclo cellulare, stress ossidativo, è stato possibile correlare l'insorgenza di questa patologia con elevati livelli di interleuchine IL-1, IL-6, IL-8, IL-10, sebbene i meccanismi sottesi all'insorgenza di questa condizione non siano ancora del tutto conosciuti (Halis e Arici, 2004). Studi recenti hanno evidenziato come alti livelli di TNF-α, IL-7, IL-10 e TGF-β1 (Transforming Growth Factor β1) siano dovuti alla over-espressione di CD33r-SIGLECS (Wang et al., 2011). Nel ciclo ovarico umano, la fase di ovulazione ha caratteristiche simili a una reazione infiammatoria. L'istamina rilasciata dalla degranulazione dei mastociti può facilitare la permeabilità dell'endotelio e aumentare il flusso di sangue all'ovario durante il processo di ovulazione. Il ligando di SIGLEC-11 si trova su mastociti umani, il che suggerisce che l'interazione tra SIGLEC-11 e i suoi ligandi sui mastociti potrebbe stimolare la secrezione di istamina prima dell'ovulazione. Un'altra citochina correlata all'ovulazione è GRO-α (CXCL1, Growth Regulated Alpha protein). Uno studio in vitro ha dimostrato che GRO-α è in grado di attirare e attivare neutrofili, così come stimolare la formazione di nuovi vasi sanguigni per il corpo luteo (Kawano et al. 2007). Enzimi proteolitici secreti dai neutrofili, come collagenasi e elastasi, possono digerire le proteine della matrice extracellulare e plausibilmente contribuire alle fasi finali di indebolimento della parete follicolare che precede l'ovulazione.
In altre patologie a carico dell'ovario, come ad esempio nei casi di PCOS (Polycystic ovary syndrome) o nei tumori, le normali funzioni ovariche possono essere alterate a seguito della presenza di alterati livelli di citochine e cellule del sistema immunitario.
Nell'ovario PCOS è stata riscontrata, ad esempio, una forte espressione di SIGLEC-11 che potrebbe essere anche coinvolto nella determinazione della atresia follicolare e della formazione dei corpi lutei, modulando la secrezione di alcune citochine. È stato dimostrato che nei fibroblasti il TGF-β1 può promuovere la biosintesi del collagene, fondamentale per lo sviluppo della fibrosi in un modello di topo (Ong et al., 2009). TNF-α potrebbe svolgere un ruolo fisiopatologico nei casi di PCOS umani grazie alla sua sovraespressione, sebbene i fattori a scatenanti questa condizione siano ancora del tutto incerti.
L'espressione di SIGLEC-11 sulle cellule stromali di ovari umani potrebbe contribuire a creare un microambiente unico di citochine o ormoni importante per lo sviluppo dei follicoli e per garantire un normale funzionamento ovarico e, allo stesso modo, per definire i cambiamenti fisiologici che portano alla insorgenza della menopausa o alla sindrome dell'ovario policistico.
Ulteriori studi sono ovviamente necessari per definire l'esatto ruolo di questo fattore, considerando però che SIGLEC-11 non è presente nei roditori e che i campioni ovarici umani sono materiale prezioso da reperire. Inoltre, sarà interessante studiare anche SIGLEC-16, espresso da una minoranza di esseri umani, ovvero un attivatore di SIGLEC che ha una sequenza N-terminale quasi identica a SIGLEC-11 (Cao et al. 2008) e che potrebbe rivelarsi la chiave necessaria per arrivare a definire i meccanismi che inducono il processo di infiammazione in diverse patologie umane.

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Biochimica dell'infiammazione

5

Highlighting Extracellular Matrix and Inflammation

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Introduction

The extracellular matrix (ECM) is a complex meshwork of molecules that constitutes the architecture of tissues. ECM is specific for each organ and changes dramatically during aging and development and it cannot be considered a passive component of the organs. The ECM is voluminous, almost insoluble and typically consists of proteins, which frequently are glycosylated and contain glycosaminoglycan (GAG) chains that are sulphated and have negative charge (proteoglycan PG). Only hyaluronan is GAG without sulphated residues and core protein and is present in ECM as polymer with a mass ranging from 0,5 x 10^6 to 3 x10^7 Dalton. The ECM exists in different biochemical and structural forms; both their individual components and three-dimensional ultrastructure interact with cells triggering signals that regulate tissue metabolism. The ECM modulates basic metabolic activities that are important in the physiopathology as well as in the early steps of inflammation including cell migration into inflamed tissues and cell activation. In chronically inflamed tissues, ECM changes composition and fragments of the ECM derived from tissue-remodelling processes can modulate immune cells activities.
In inflammatory processes, numerous extracellular proteins and GAGs undergo limited enzymatic cleavage resulting in the release of fragments with biological activities that are usually different from those of the full-length molecules. These fragments with biological activities are usually masked in full-length form. Various events as denaturation, mechanical stress or reactive oxygen species (ROS) can expose bioactive matricryptic sites in the extracellular matrix (ECM) (1). The biologically active fragments are defined matrikines and matricryptins and represent defined bioactive fragments released from the ECM by enzymatic degradation.

Extracellular biologically active fragments

Maquart et al. (2) defined matrikines in 1999 as 'ECM-derived peptides able to regulate cell activity'. Swindle et al. (3) used the term 'matrikine' to describe low-affinity ligands for growth factor receptors. Matrikines were defined

in those papers 'as signalling elements that exist as subcomponents of ECM proteins and bind to cell surface receptors that belong to the cytokine, chemokine, ion channel or growth factor receptor family'. These ligands are encrypted within matrix components and modulate cellular responses by using growth factor receptors. Davis et al. (4) proposed the name 'matricryptin' for 'enzymatic fragments of the ECM containing exposed matricryptic sites'. This term was restricted to biologically active fragments from the ECM exposing functional matricryptic sites not normally exposed in the full-length molecules. The definition takes into account the molecular sources of matricryptins, which can be insoluble ECM molecules, matricellular proteins and plasma derived ECM molecules. Peptides released from growth factors, chemokines and cytokines are not considered as matricryptins or matrikines.

Although matricryptins may be considered as a subgroup of matrikines, the distinction between both terms is sometimes not easy to define. Schor and Schor (5) refer to matrikines as 'proteinase-generated fragments of matrix macromolecules that display cryptic bioactivities not manifested by the native, full-length form of the molecule', which combines criteria defining both matrikines and matricryptins. Arroyo and Iruela-Arispe (6) define matrikines as 'fragments of ECM molecules with biological functions distinct from those of the parental protein', and Tran et al. (7) distinguished the 'natural matrikines, which signal directly from the extracellular milieu, and cryptic matrikines or matricryptins that require limited proteolysis to act as ligands and fulfil their biological roles'. The C-terminal fragment of matrix metalloproteinase-2 (MMP-2) inhibits MMP-2 activity and angiogenesis and work as cryptic sites in the full-length enzyme (8).

The fragments of ECM regulators and ECM-affiliated proteins (mucins, galectins, semaphorins) (9) can be considered as matricryptins. Matricryptines are also the ectodomains of membrane collagens XIII, XVII, XXIII and XXV (10), which are shed from the cell surface and released into ECM. The ectodomain of collagen XIII modulates cell adhesion, migration and proliferation, whereas increased shedding of collagen XVII decreases keratinocyte motility. The shed of syndecans 1–4 ectodomains influences cell adhesion (11). The matrikine metastatin is proteolytic fragment of the link protein and the aggrecan core protein that inhibits angiogenesis and tumor growth (12). Oligosaccharides cleaved from hyaluronan (13) are matricryptins.

Sources of ECM bioactive fragments

Collagens and proteoglycans (10), elastin (14) and laminins (15) are major sources of matricryptins. Matricellular proteins as SPARC and osteopontin are additional sources of bioactive fragments. Furthermore, a full-length biomolecule and its matricryptins may have opposite activities as reported for hyaluronan, which exerts anti-angiogenic properties as large size polymer but its fragments are pro-angiogenic. The size and source of matrikines and matricryptines are reported in table 1.

Tab. 1

	MATRIKIN/ MATRICRYPTIN	**MOLECULAR MASS kDa**
AGGRECAN core protein	metastatin	85-38
COLLAGEN TYPE IV Alpha1 chain	arresten	26
COLLAGEN TYPE IV Alpha2 chain	canstatin	24
COLLAGEN TYPE IV Alpha3 chain	tumstatin	27
COLLAGEN TYPE IV Alpha4 chain	tetrastatin	25
COLLAGEN TYPE IV Alpha 5 chain	Pentastatin (1-3)	2,5/2,4/2,1
COLLAGEN TYPE IV Alpha 6 chain	Hexastatin (1-2)	2/2,5
COLLAGEN VI Alpha 3 chain	endotropin	5,8
COLLAGEN XIII Alpha 1 chain	Ectodomain XIII	240
COLLAGEN XV Alpha 1 chain	Restin (1-4-)	21/20/19,4/18,4
COLLAGEN XVII Alpha 1 chain	Ectodoman coll XVII	120
COLLAGEN XVIII Alpha 1 chain	endostatin	21
COLLAGEN XIX Alpha 1 chain	NC-1 domain	2,2
COLLAGEN XXIII Alpha 1 chain	Ectodomain coll XXIII	180
COLLAGEN XXV Alpha 1 chain	Ectodomain coll XXV	158
FIBRONECTIN	Anastellin Fibstatin	10 29
LAMININ	Laminin peptides	
MMP-2	PEX	24
PERLECAN	Endorepellin LG-3	85 21
PROCOLLAGEN-C	CUB-1, CUB-2	30
TENASCIN-C	Ten 1-2, Ten 11-14	10/ 3,4
SYNDECAN-1	Ectodomain syndeca 1	24
SYNDECAN-2	Ectodomain syndeca 2	14
SYNDECAN-3	Ectodomain syndeca 3	39
SYNDECAN-4	Ectodomain syndeca 4	14
TROPOELASTIN	elastokines	0,5-8

Bioactive fragments are released from full-length proteins by limited proteolysis catalysed by proteases as cathepsins (16) and MMPs [MMP-2, MMP-7 and MMP-13] for endostatin (17), MMP-9 for tumstatin (18), MMP-7, MMP-9, MMP-12 for elastokines. The matricryptin endorepellin is not the final product but it can be further processed by metalloprotease (Bone Morphogenetic Protein-1, BMP-1) to give the LG3 domain that is a smaller matricryptin, (19). The ectodomains of membrane collagens are shed by ADAMs [ADAMs 9 and 10 for collagen XVII] or by the furins for collagens XIII, XXIII and XXV. Dispase might release fibstatin from fibronectin. Mechanical forces, as shown for endostatin in tendon cells (20) may modulate the release of proteolytic ECM fragments.

Molecular functions and biological activities of ECM fragments

The matricryptins can be released in vivo in body fluids and tissues, examples are endostatin, the LG3 domain from endorepellin, restin and tumstatin. Some matricryptins/matrikines regulate several biological processes in health and pathology. Some authors consider endogenous ECM fragments released in the bloodstream as hormone-like proteins as regulate cell behaviour. ECM fragments regulate angiogenesis, tumour growth and metastasis, lymphangiogenesis, inflammation. Obesity and adipogenesis also seem to be influenced by ECM fragments, in fact endostatin induces weight reduction in a murine model of obesity and regulates adipose tissue mass. Elastin peptides regulate insulin resistance in mice and endotrophin, a cleavage product of collagen VI secreted by adipocytes, may play a role in obesity-related cancers. Endotrophin also promotes tumour progression and tissue fibrosis, whereas endostatin has an opposite effect. Matrikines of collagens IV and XVIII (endostatin) play a role in synapse formation. Furthermore, endostatin inhibits neurite outgrowth, and endorepellin may have a neuroprotective effect following stroke. In contrast, other matricryptins, such as endostatin and anastellin, are able to form amyloid fibrils in vitro and might contribute to the formation of amyloid deposits in neurodegenerative diseases. At the molecular level, ECM fragments modulate cell adhesion, proliferation, migration and apoptosis and may induce autophagy as shown in endothelial cells for endostatin; they are chemotactic for fibroblasts, endothelial cells and monocytes [elastin peptides] and inhibit MMP enzymatic activity as reported for endostatin or protease activation/secretion as described for laminin peptides. ECM bioactive fragments bind to growth factors such as nerve growth factor [endostatin] and FGF-2, VEGF and PDGF [fibstatin].

Receptors mediate biological activities of ECM fragments

Integrins, heparan sulphate proteoglycans and growth factor receptors mediate most biological activities of the ECM fragments. Endostatin, canstatin, tumstatin, tetrastatin and the PEX domain of MMP-2 bind to the avb3 integrin. Other ECM fragments interact with b1 integrins, including anastellin, arresten, endostatin [a5b1 and a3b1], endorepellin [a2b1 and a5b1], the ectodomain of collagen

XIII [a1b1] and the cell adhesion domain of the ectodomain of collagen XVII. Angiogenic laminin peptides (C16 and A13) also bind to avb3 and a5b1 integrins. Nucleolin, a functional receptor of endostatin, facilitates its internalization in endothelial cells in association with a5b1 integrin and the urokinase plasminogen activator receptor. Cell surface heparan sulphate proteoglycans [glypicans 1 and 4] may bind ECM fragments to the cells via their heparan sulphate chains as shown for anastellin, arresten and endostatin. Several ECM fragments bind to growth factor receptors, VEGFR2 [endostatin and endorepellin] and the EGF receptor. Some ECM fragments have a specific receptor, the elastokines bind to a spliced variant of bgalactosidase and hyaluronan oligosaccharides bind to CD44.

Dual activities of ECM fragments

Two anti-angiogenic matricryptins, endostatin and endorepellin, have been reported to exert pro-angiogenic activities depending on the context and on the cell types. Endorepellin acts as a proangiogenic factor following stroke and endostatin is proangiogenic for embryonic stem cell endothelial-derived cells. The biological role of EGF-like repeats of tenascin also depends on the molecular context and on their mode of presentation to the EGF receptor. Several EGF-like repeats of tenascin-C elicited mitogenesis in an EGF receptor-dependent manner, and the soluble 14th EGF-like repeat of human tenascin-C preferentially activates cell migration over proliferation.

Size and structure of ECM fragments plays a role

The size of ECM fragments ranges from few amino acids [the RGD tripeptide] to more than 700 amino acids [endorepellin], but the distinction between the smallest bioactive ECM fragments and bioactive oligopeptides is still elusive. The size of ECM fragments regulates their biological activities. Hyaluronan oligosaccharides in angiogenesis and wound healing have an opposite effect compared to large hyaluronan chain.

ECM biological fragments as future therapeutical agents

The translational potential of the ECM has already been investigated for controlling angiogenesis. ECM bioactive fragments are potential drugs, but their translation into the clinics is challenging. Experimental models of cancer, wound healing, fibrosis and infectious diseases are established to test the possible use of ECM fragments in clinic. Fragments with anti-angiogenic, antitumoral and/or antimetastatic properties gave promising results in these preliminary studies. The antifibrotic effects of ECM fragments can be useful in fibrotic diseases, hypertrophic scars and keloids. The neuroprotective fragment endorepellin is used in experimental stroke treatment (21), and showed an anti-amyloid potential (22). Large body of literature shows that ECM fragments used in combination do not have a synergistic effect. However, the combination of ECM fragments with

chemokines may have some synergy (23), and the association of anti-angiogenic and antitumoral ECM fragments with radiotherapy or chemotherapy potentiates their effects (24). The combination of the anti-angiogenic endostatin with a small interfering RNA-targeting signal transducer and activator of transcription 3 (Stat3) was able to inhibit its signalling improving antitumoral effects (25). The therapeutically active doses of ECM fragments are difficult to define as many angiogenesis inhibitors display biphasic response curves (26). Another challenge is that several ECM fragments contain further cryptic sequences that may display opposite activities when used in vivo. This finding was reported for the anti-angiogenic endostatin, which contains embedded a proangiogenic sequence. Several phase II studies with endostatin and one of its derivatives, Endostar, alone or in combination with chemotherapy have shown to provide benefits in breast cancer patients (27) and may improve survival of patients with non-small-cell lung cancer and metastatic melanoma (28).

Perspectives

The building of the interaction network of endostatin and of the N-terminus of procollagen C-proteinase enhancer-1, allows us to define new extracellular molecular links between angiogenesis and neurodegenerative diseases (29). A systems biology approach is required to account for the complexity of the regulatory processes mediated by ECM bioactive fragments and to predict potential side effects of ECM fragments tested as drugs. Several approaches are used to search for new matricryptins, either by looking in tissues or biological fluids for fragments released from ECM and ECM-associated proteins by limited proteolysis at known enzymatic cleavage sites or by investigating the biological functions of protein termini at the proteomic scale (30).

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6

Role of protein postranslational modifications in inflammation

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Proteins can be widely modified once translated or during translation. Posttranslational modifications (PTM) increase the functional diversity of the proteome by the covalent addition of functional groups or proteins, proteolytic cleavage of regulatory subunits or degradation of entire proteins. These modifications include phosphorylation, glycosylations (including O-, N-, and O-GlcNacylation), ubiquitination, nitrosylation, methylation, acetylation, lipidation and proteolysis and influence almost all aspects of normal cell biology and pathogenesis. All amino acids can be modified in vivo (Ribet et al., 2010); many PTM are finely regulated by the cell, but others are driven by non-enzymatic reactions and take place when a particular metabolite accumulates in the cells. Therefore, identifying and understanding PTMs is critical in the study of cell biology and disease treatment and prevention.
In the last fifty years, changes in lifestyle, including excessive energy intake and consumption of food enriched in saturated fat, combined with the lack of physical activity, have led to a dramatic increased prevalence of pathologies such as diabetes, obesity, and atherosclerosis. It is now generally accepted that these pathologies are associated with a low-grade chronic inflammation (Hotamisligil 2006) that causes complications such as nephropathy, neuropathy, retinopathy, and atherosclerosis. In the classic literature, inflammation is described as the principal response of the body invoked to deal with injuries, the hallmarks of which include swelling, redness, pain and fever (tumor, rubor, dolor and calor). This often short-term adaptive response is a crucial component of tissue repair and involves integration of many complex signals in distinct cells and organs. However, the long-term consequences of prolonged inflammation are often not beneficial. This certainly seems to be the case in metabolic diseases. Although many of the same mediators are involved in obesity and diabetes, few, if any, of the classic features of inflammation have been observed. Therefore, it would be useful to set out a distinct form of injury response or subclass of inflammation - sometimes referred to as ‘low-grade’ or ‘chronic’ - or to describe an altogether separate state with a new term, perhaps ‘metaflammation’ (metabolically triggered inflammation). This condition is principally triggered by nutrients and metabolic surplus, and

engages a similar set of molecules and signaling pathways to those involved in classical inflammation.

The relationships between metabolic diseases and inflammatory processes are complex. The most critical processes to species survival are the ability to withstand starvation and the capacity to mount an effective immune response to pathogens. The combination of these traits is likely to have given rise to a biological organization that is highly capable of processing and storing energy and is also equipped with a powerful, and perhaps at times overly sensitive, immune response. There is also an intimate relationship between the immune and metabolic response systems that has many evolutionary underpinnings. In fact, the functional units that control key metabolic and immune functions in higher organisms have evolved from common ancestral structures. One such structure is the *Drosophila* fat body, which incorporates the mammalian homologues of the liver and the haematopoietic and immune systems as well as the equivalent of mammalian adipose tissue (Sondergaard 1993; Tong et al., 2000). Intriguingly, it is possible to imagine a situation in which common or overlapping pathways regulate both metabolic and immune functions through common key regulatory molecules and signaling systems. This might allow nutrients to act through pathogen-sensing systems such as Toll-like receptors (TLRs), giving rise to metabolically or nutritionally induced inflammatory responses (Shi et al., 2006).

Low-grade inflammation is characterized by an abnormal cytokine production. Thus, it has been demonstrated that the adipose tissue of obese individuals produce higher levels of the pro-inflammatory cytokine tumor-necrosis factor α (TNFα) and other pro-inflammatory factors such as interleukin 6 (IL-6). The excessive amount of nutritional lipids might have a role not only in the pathogenesis of obesity-associated insulin resistance but also in the chronic inflammation associated with this condition. Indeed, free fatty acids can activate the lipopolysaccharide (LPS) receptor TLR4 and induce the production of pro-inflammatory cytokines by macrophages. Not only lipids but also high-glucose concentrations are involved in inflammatory processes and high glycemic index diets appeared to play a key role in the establishment and persistence of inflammation (Esposito et al., 2002; Dickinson et al., 2008).

Hyperglycemia can induce several perturbations at biochemical level involving increased polyol pathway flux, increased advanced glycation end-product (AGE) formation; activation of protein kinase C (PKC) isoforms; and increased hexosamine biosynthetic pathway (HBP) flux (Brownlee 2001). This latter pathway is very interesting as can work as a nutrient sensor and participate to protein O-GlcNacylation (Fig. 1). In fact, the flux of nutrients that enters in the HBP is usually limited (2-5% of the total) but, in hyperglycemic conditions the excess of glucose brings to the formation of UDP-GlcNAc via the HBP (Copeland et al. 2008). O-GlcNac transferase (OGT) is an intracellular enzyme characterized by a low affinity for UDP-GlcNAc. Therefore an increment of UDP-GlcNac concentration leads to the activation of OGT. Such enzyme catalyzes the transfer of GlcNAc form the UDP-sugar to the hydroxyl group of serine and threonine. Such intracellular glycosylation is named O-GlcNAcylation (Hart et al., 2007).

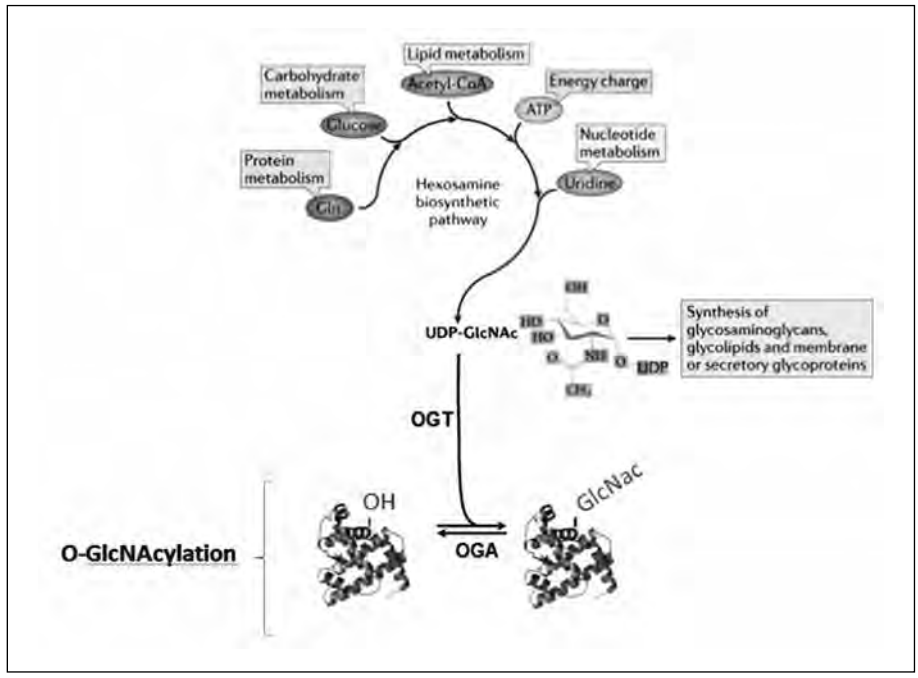

Fig. 1 - Schematic representation of the hexosamine biosynthetic pathway (HBP). UDP-GlcNAc levels can greatly fluctuate in function of protein, carbohydrate, lipid, energy, and nucleotide metabolisms making the HBP an efficient energy sensor. O-GlcNAcylation is the reversible addition of β-D-N-acetylglucosamine (from UDP-GlcNAc) to serine or threonine residues of nuclear and cytoplasmic proteins. OGT, O-GlcNAc transferase; OGA, O-linked N-acetylglucosaminase (from Hannover et al., 2012).

O-GlcNAcylation controls many cellular processes and is involved also in diabetes, cardiovascular diseases, tumors and inflammatory processes (Slawson et al., 2010). O-GlcNAcylation is a very dynamic process, not also because O-GlcNAc can be easily hydrolyzed from proteins by O-GlcNAcase (OGA), but also because O-GlcNacylation can compete or control with protein phosphorylation (Zeidan et al., 2010). Protein modified by O-GlcNAcylation can alter their, activity, localization, stability, and interaction with other molecules; interestingly, at nuclear level, O-GlcNAcylation can greatly alter gene expression by modulating transcription factors or RNA polymerase 2 activity, or altering chromatin structure by controlling epigenetic machinery (Hannover et al 2012; Vigetti et al., 2014).
The transcription factor nuclear factor κB (NFκB) is involved in a large number of cell functions including apoptosis, cell survival, and differentiation, and is critical to immune response and inflammation. NFκB family comprises five proteins, p65 (RelA), RelB, c-Rel, p105/p50 (NFκB1), and p100/52 (NFκB2) that associate to form distinct homo and hetero-dimeric complexes. In non-stimulated cells, NFκB is inactive and is retained in the cytoplasm by the inhibitor of κB (IκB). Upon stimulation by pro-inflammatory cytokines, LPS, or growth factors, IκB is phosphorylated by the IκB kinase (IKK). This phosphorylation leads to IκB ubiquitination and proteosomal degradation. Free NFκB can then translocate into the

nucleus to activate its target genes. O-GlcNAcylation of p65 (RelA) (Baudoin et al., 2015) associated with hyperglycemia has been shown to finely regulate NFκB transcriptional activity through different mechanisms that can compete and co-operate with other NFκB PTM as phosphorylation and acetylation. Interestingly, O-GlcNAcylated NFκB can exert pro- and anti-inflammatory properties, suggesting a complex regulation of inflammation by O-GlcNAc.

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7

Extracellular Matrix Components and Megakaryocytes in Bone Marrow Fibrosis

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Megakaryocyte function within the bone marrow environment

Bone marrow (BM) is a complex tissue where blood cells are produced and released in the blood stream (1). Packaged into the cavity of bones, the BM remains one of the most difficult organs to study and data on its structural composition have been primarily derived from *in vitro* long-term cultures of BM-derived cells and from immunofluorescence microscopy analysis (2). The BM is a tridimensional network of branching sinusoids surrounding islets of hematopoietic cells immersed in a mesh of extracellular matrix components (ECMs) and soluble/humoral factors (2). ECMs confer unique physical, biochemical and biomechanical properties that are essential for regulating cell behavior within the BM environment. The physical properties of the ECMs refer to their rigidity, porosity, insolubility, spatial arrangement and topography, and other physical features that together determine their role in scaffolding to support tissue architecture and integrity.
In the BM, the mechanisms of single cell differentiation and transit to the bloodstream must be tightly regulated to satisfy the physiological blood cell requests and supply. Recent advancements in the characterization of the BM structure and function, have favored the identification of specialized microenvironments linked to the direction of hematopoietic stem cell (HSC) quiescence, differentiation or mobilization (3). In this regard, cell composition and localization have permitted researchers to discriminate a peri-vascular niche mainly composed of endothelial, $CXCL12^+$ reticular cells and $Nestin^+$ mesenchymal stromal cells from an endosteum-associated niche occupied by osteoblasts and osteolineage cells, including the more recently identified arteriolar niche sustained by $NG2^+$ (CSPG4) pericytes. Despite the fact that a coordinated interaction and connection between these niches is indispensable for efficient HSC differentiation and BM function, the anatomical definition of those districts as separated or overlapping entities is still debated (4). Within this context, megakaryocytes (Mks) migrate, while differentiating from HSCs under the effects of thrombopoietin (TPO), from the endosteal to the vascular niche where platelets are shed into the bloodstream (5). To assemble and release platelets, Mks follow a maturation program that accumulates in the conversion of the bulk of their cytoplasmic into multiple long processes called proplatelets, which are extended directly through the endothelium

of marrow sinusoids. As a consequence of their function, Mks are mainly associated with the vascular BM niche and this hypothesis was recently confirmed by intravital microscopy fluorescence in mouse BM (6). The positioning of Mks in close proximity of BM sinusoids relies on chemokines that are released by several BM cell types, such as endothelial and perivascular stromal/mesenchymal cells. Interestingly, *ex vivo* analysis of Mk localization in murine BM demonstrated that these cells can be differently located in the BM and associated with endosteum, arterioles and sinusoids based on their maturation stage (7).
While migrating between BM endosteal and vascular niches, Mks interact with different cellular components of the BM. Several studies have demonstrated that Mks establish interactions with both stromal lineages arising from the mesenchymal stem cells as well as lineages of hematopoietic origin. As an example, after transplantation into irradiated mice, HSCs lodge preferentially near Mks, and inhibition of Mks impairs HSC engraftment (8). Further, Mks are required for osteoblast expansion after irradiation, suggesting that Mks regulate HSC engraftment indirectly by expanding the osteoblastic niches (9). Recently, two studies revealed that hematopoietic-derived Mks also contribute directly to the HSC niche, regulating HSC quiescence and function by producing chemokines and growth factors that control HSC proliferation (10, 11). All together these data demonstrate that Mks are not solely involved in platelet production but also in the maintenance of BM function.

Megakaryocytes and extracellular matrices

The BM extracellular microenvironment is composed of structural fibrils and extracellular matrix components. The most common structural fibrils in the BM are collagen, reticulin, laminin and fibronectin (12), embedded in a matrix of glycosaminoglycans and glycoproteins, most of which have not been well characterized. *In vivo*, Mks interact with ECM components, that play the double function of being a regulator of platelet formation and a barrier that Mks need to rearrange and traverse in order to extend proplatelets. Interestingly, individual ECM components were proven to play a role in the regulation of Mk development *in vitro*. Fibronectin (FN) was shown to regulate Mk maturation and proplatelet extension, while collagen type III and type IV were demonstrated to support proplatelet formation. In contrast, collagen type I is a physiological inhibitor of platelet release *in vitro* (7, 13). Overall these studies suggest that, although platelet biogenesis is a cell-autonomous process, collagen type I inhibits proplatelet formation by preventing premature platelet release in the BM cavity, while collagen type IV and FN support proplatelet formation at the sinusoids. Mks are directly involved in matrix deposition and remodeling, as confirmed by their role in FN fibrillogenesis and the expression of matrix cross-linking enzymes, such as lysil oxidase and factor XIIIa (14, 15). Mks also possess collagenase activity, as they express several MMPs, such as MMP-2, MMP-9, MMP-14, MMP-24, MMP-25, and assemble podosomes that rearrange the ECM through proteolytic activity essential in the dynamic of Mk-matrix component interactions. Thus Mk-

ECM interaction in the BM seems to be a key step in the regulation of platelet generation as well as in the maintenance of BM homeostasis.

Bone marrow fibrosis and related pathologies

A wide variety of benign and malignant disorders are characterized by an increase in BM stromal fibers (12). These fibers are commonly composed solely of reticulin fibers but may also include collagen fibers. Immunohistochemical detection of reticulin with silver-based stains and collagen with trichrome stains represent routine stains performed on BM biopsy specimens in diagnostic laboratories. Two slightly different, but widely supported grading scales for these stains have been created (16, 17). The original Bauermeister scheme has been simplified into a five grade system, while, the more recent Thiele scale includes only four categories. Both of these grading scales take into account the type of fibers seen (reticulin or collagen) and the overall amount of fibrosis. A reactive (non clonal) proliferation of fibroblasts leads to the increase of ECM fibrils and to the development of BM fibrosis. BM fibrosis may occur in association with several haematological malignancies, such as myeloproliferative neoplasms, myelodysplastic syndromes, acute leukemia and metastatic tumors. However, fibrosis may be also present in non-neoplastic conditions, especially inflammatory diseases, autoimmune disorders (e.g. Systemic Lupus Erythematosus), BM regeneration after chemotherapy or irradiation and metabolic disorders.

Most diseases with increased BM fibrosis display abnormalities of the number and/or function of Mks in addition to a perturbed balance of fibrotic/immunological factors that leads to abnormal function of the BM stromal cells and uncontrolled ECM release (18). The BCR-ABL-negative Myeloproliferative Neoplasms (MPNs) are a group of clonal hematological malignancies originating from HSCs, leading to an increase in mature blood cells in the peripheral blood. BCRABL-negative MPNs have been classified by the World Health Organization (WHO) as a single entity, however they comprise three clinically defined disorders called Polycythemia Vera (PV), Essential Thrombocytemia (ET) and Primary Myelofibrosis (PMF), although the boundaries between them are sometimes difficult to assign (19). In MPNs, fibrosis has been described as a 'reactive process mediated by cytokines that are produced by the cellular components of the hematopoietic clonal proliferation' (19). Three different mutations affecting the TPO receptor (MPL) activity have been described (20, 21). In particular, driver mutations have been identified in genes encoding the Janus Kinase-2 (*JAK2)*, a kinase underlying TPO signaling pathway, the TPO receptor MPL and the recently described mutations in *CALR,* the calreticulin gene (22). In general, BM from MPNs patients presents neoplastic proliferation and maturation of erythroid, megakaryocytic and granulocytic elements. In particular, Mks are increased in number and have characteristic morphological abnormalities, such as hyperlobated nuclei and hyperplasia. Nearly 95% of PV and 50% of ET and PMF present a somatic point mutation in the gene encoding for JAK2 (JAK2V617F). However, only PMF patients show increased deposition of reticulin and collagen fibers.

BM fibrosis is the major responsible for the final BM failure, with consequent splenomegaly because of the appearance of extramedullary hematopoiesis. MPN oncogenes cannot singlehandedly explain the wide biological and clinical heterogeneity of these diseases. The microenvironment where the mutated hematopoietic cells reside holds cues in the pathogenesis of MPNs and the progression to fibrosis.

Megakaryocytes contribute to bone marrow fibrosis

When an increase in BM reticulin staining does occur, it is usually accompanied by an increase in BM Mks, often morphologically atypical. Besides BM a variety of organs including lung, liver, kidney, intestine, heart, skin may develop fibrotic disease. It is currently assumed that ECM components are mainly synthesized by resident stromal cells and that the underlying mechanism of fibrotic reactions, leading to progressive organ imbalance, involves the over-stimulation of these resident stromal cells, namely mesenchymal cells and myofibroblast, to produce and deposit collagens and other ECM components. Our group has identified Mks as ECM-producing cells, based on their ability to synthesize fibronectin, type IV collagen and laminin, both *in vitro* as well as *in vivo*, although the exact function of the self-produced ECM components is still unclear. Evidence suggests that expression of many of those ECMs is not related to the physiological production of platelets but is increased concomitantly with the regeneration of BM environment following myelosuppression, thus reinforcing the role of Mks in the control of BM homeostasis and function (7, 23).

ECM component synthesis is modulated by the effect of various profibrotic cytokines, particularly by transforming growth factor-β1 (TGF-β1), whose principal source, in the BM, are Mks (24). TGF-β1 is the first member identified of pleiotropic cytokines involved in normal tissue repair and development. Although expressed by all cell types, it is especially abundant in platelets. TGF-β1 activity is regulated by proteases that convert the latent complex into an active form, thus inducing the expression of both extracellular matrix components (collagen type I and FN) and their cognate receptors on target cells and its sustained production induces fibrosis in numerous organs, including myelofibrosis (25). The role of TGF-β1 and its downstream signaling is suggested by the evidence that development of myelofibrosis in wild type mice treated with high doses of TPO is associated with high TGF-β1 content in extracellular fluids of both BM and spleen, and that TGF-β1 deficient mice over-expressing TPO are fibrosis resistant as compared to wild type mice. In parallel to these facts, it has been also reported that Mks from PMF patients synthesize and release higher amount of both latent and bioactive TGF-β1, and that this behavior is highly correlated with BM fibrosis grade of investigated patients (26-28). Finally, we recently demonstrated that in human Mks synthesis of fibronectin, collagens type III and type IV, is promoted by self-produced TGF-β1, which is actively secreted by Mks under the control of TPO, thus identifying a potential autocrine loop which might participate into the aberrant megakaryopoiesis and ECM deposition found in BM of PMF patients (29).

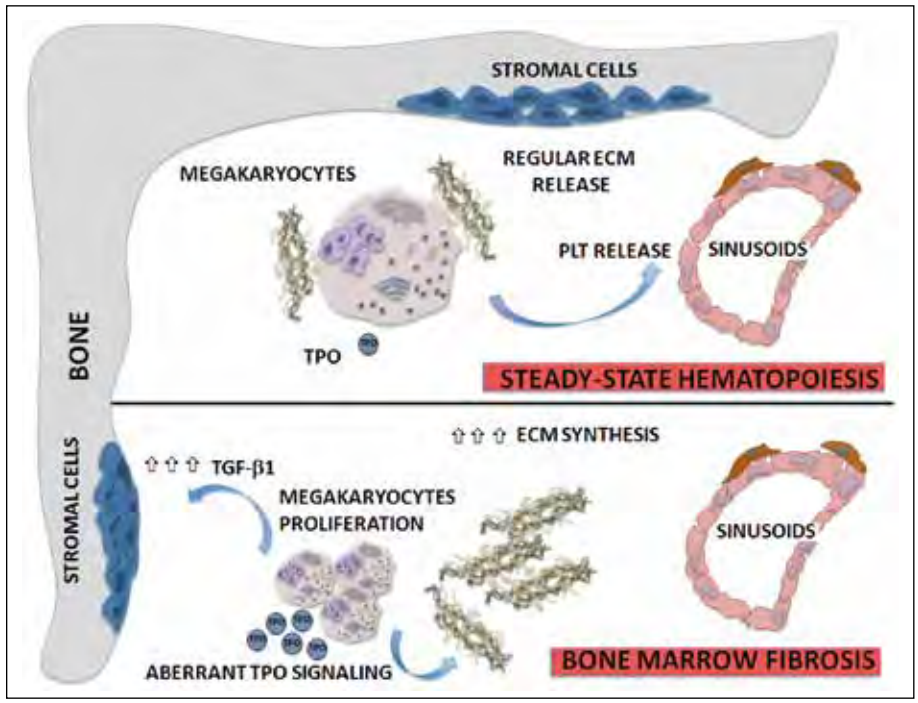

Fig. 1 - Schematic representation of megakaryocyte contribution to the bone marrow environment in physiology or fibrosis progression. During steady-state hematopoiesis, Mks are responsible of platelets production and ECM remodeling within BM environment (Top Panel). However, aberrant TPO signaling, as in disease, induce Mk proliferation, ECM components and pro-fibrotic factor (e.g. TGF-β1) release, which in turn promote sclerosis of BM tissue (Lower Panel).

Cellular fibronectin is a new regulator of BM inflammation and fibrosis

To date the etiological mechanisms of BM fibrosis remain unclear and in the case of MPNs, the same genetic mutation might result in a different BM phenotype with a different level of fibrosis, suggesting that several cell types, cytokines and growth factors are involved in fibrosis development. In MPNs, fibrosis progression is not solely due to HSC proliferation but also to the loss of tightly balanced response between inflammatory and fibrogenic signals. In particular, the inflammatory component of MPNs is due to the release of a large number of inflammatory cytokines by malignant and non-malignant cells due to an aberrant activation of the JAK/STAT signaling pathway.

FN, a glycoprotein of about 220 kDa produced by many types of cells, forms ECM when secreted (30). FN mRNA has three alternative splicing sites (termed EDA, EDB and IIICS in human or EIIIA, EIIIB and V in mice) that allow 20 different isoforms of FN mRNA. EIIIA and EIIIB exons are included or excluded from the FN mRNA by exon skipping (31). Plasma FN (pFN) lacks both EIIIA and EIIIB segments and is a soluble form secreted by the hepatocytes, while cellular FN (cFN) contains variable proportions of EIIIA and EIIIB segments

and is found as fibrils in the ECM (32). The levels of expression of the spliced forms and their relative proportion change during embryonic development and in pathological processes. Specifically, EIIIA and EIIIB exons tend to be excluded in most adult tissues, whereas they are largely included in a TGF-β1-dependent manner during events that involve tissue rearrangements, such as embryogenesis and wound healing, resulting in a tissue-dependent, temporally regulated and cell type-specific expression. FN containing EIIIA domain presents peculiar biochemical properties as compared to the unspliced form:

1) fibrogenic, as the EIIIA-containing isoform become predominantly expressed during wound healing to enhance lypocyte and fibroblast differentiation into α-smooth muscle actin+ (αSMA) myofibroblasts (33),
2) pro-inflammatory, as inclusion of EIIIA domain activates Toll Like Receptor 4 (TLR4), involved in the defense of the innate immune response following recognition of pathogens associated molecular patterns resulting in NF-κβ-dependent cytokines release (34),
3) pro-thrombotic, as EIIIA FN was shown to promote agonist-induced platelet aggregation and thrombus formation *in vivo* through TLR4 dependent mechanisms (35).

More than three decades ago, abnormal forms of FN were found in plasma samples of PMF patients by immunoassays, while recently, increased synthesis of FN was detected in BM-derived mesenchymal stem cells (MSCs) of pre-fibrotic and PMF patients. Thus, inflammatory properties of ECM may concur to alter the BM environment to render it more permissive for mutated HSCs to proliferate in diseases.

Concluding Remarks

In conclusion, Mks actively interact with the different components of the BM ECM components that contribute to the regulation of platelet production and BM homeostasis. Many aspects of these fascinating processes have still to be explained and new insight into the molecular events driving fibrotic progression will have a major impact on the development of more appropriate therapeutic strategies tailored to the different phenotypes of BM disease.

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8

Le prospettive della lotta al cancro e della sua prevenzione

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Continua a essere un flagello, responsabile ogni anno di circa 1/3 delle cause di morte. Tuttavia, oggi il cancro fa meno paura. Gli anni recenti, infatti, sono stati caratterizzati da importanti progressi dal punto di vista sia della diagnosi sia della cura: le nuove terapie hanno permesso di trasformare alcuni tumori in malattie croniche, riuscendo a prolungare la sopravvivenza ed assicurando una buona qualità di vita al paziente.
Questo perché stiamo conoscendo sempre meglio il cancro e i suoi punti deboli: grazie ai progressi della Ricerca, sono state svelate le caratteristiche essenziali delle cellule tumorali - riassunte da Douglas Hanahan e Robert Weinberg, sulla rivista scientifica *Cell*, per la prima volta nel 2000 e successivamente nel 2011 - e l'importanza del microambiente in cui il cancro si sviluppa. Da queste conoscenze sono derivate nuove cure, e in futuro ci aspettiamo ne derivino sempre di più.
Chirurgia e radioterapia rappresentano il fondamento della terapia dei tumori: grazie ai continui progressi e allo sviluppo di tecnologie sempre meno invasive e più mirate, di per sé sarebbero sufficienti a curare i tumori - per lo meno quelli solidi - se questi fossero una malattia localizzata. Purtroppo, però, una delle caratteristiche peculiari del cancro è la sua capacità di diffondersi nell'organismo formando metastasi. Per combatterlo, quindi, dobbiamo utilizzare armi diverse. Fra le terapie e il cancro è in atto una vera e propria "rincorsa": la cellula tumorale è un bersaglio mobile, geneticamente instabile e perciò capace di mutare diventando, gradualmente, resistente alle terapie. Anche le terapie, però, evolvono in continuazione. Questa continua ricorsa tra i farmaci e la cellula cancerosa, seppur ancora lontana da una soluzione definitiva, ha consentito di migliorare radicalmente le prospettive di vita dei pazienti.
Sul fronte dei farmaci, è fondamentale il ruolo dei chemioterapici: alcuni grandi successi della terapia, come la guarigione dalla leucemia linfoblastica del bambino nell'80-90% dei casi, sono stati possibili grazie ad un uso sempre più intelligente di queste "vecchie" armi. Più "nuove" sono invece le *target therapies,* terapie mirate a colpire specifiche alterazioni genetiche che sostengono la trasformazione delle cellule, da normali a tumorali. Il caso più eclatante è *Imatinib* per la leucemia mieloide, un farmaco mirato contro il riarrangiamento di due cromosomi che danno luogo al cromosoma *Philadephia*. Grazie alle nuove tecnologie di

caratterizzazione del genoma stiamo andando verso la cosiddetta *precision medicine*, un approccio basato sulla definizione delle alterazioni genetiche dei diversi tipi di tumore, e sul successivo utilizzo di farmaci mirate ad esse.
Ma la più grande novità - e speranza - nella lotta al cancro è l'immunoterapia, che rappresenta l'avverarsi di un sogno lungo 100 anni: oggi, le armi del nostro sistema immunitario si sono affiancate con successo alle terapie più tradizionali. Gli anticorpi, innanzitutto, che dotati di grande specificità - come missili mirati contro le cellule tumorali - hanno rivoluzionato la cura dei linfomi e di alcuni tumori solidi come mammella e polmone. In futuro ci auguriamo migliorino sempre più la vita dei pazienti: tra i nuovi farmaci in sperimentazione, uno su tre è un anticorpo. L'ultima frontiera, poi, è coniugare agli anticorpi i farmaci chemioterapici, veicolandoli direttamente contro il cancro e riducendone la tossicità sui tessuti sani.
Ancora, dalla consapevolezza che il sistema immunitario viene come corrotto o addormentato dal cancro, stanno derivando approcci terapeutici mirati a togliere alle nostre difese i "freni" che il tumore attiva. Recentemente è stato approvato l'uso clinico di anticorpi che bloccano alcuni di questi "freni molecolari" (CTLA4, PD1 e PDL1) contro il melanoma, e a breve ci attendiamo l'entrata in clinica di ulteriori anticorpi mirati contro freni diversi.
Anche le cellule dell'immunità sono entrate a far parte dell'arsenale terapeutico contro i tumori. Siamo capaci di prelevarle, farle crescere, educarle ad un determinato scopo e poi reinfonderle nei pazienti: le terapie cellulari stanno muovendo i primi passi in clinica con risultati incoraggianti nei tumori ematologici. Infine, abbiamo imparato ad utilizzare i vaccini: sono già realtà quelli preventivi (contro l'epatite B e i cancri del fegato causati dal virus che ne è responsabile, e contro il Papilloma virus che provoca il tumore della cervice uterina), mentre quelli terapeutici sono una speranza su cui si sta lavorando in tutto il mondo.
I vaccini richiamano la nostra attenzione su un'altra arma fondamentale nella lotta contro i tumori: la prevenzione. L'unico strumento a nostra disposizione in grado di eliminare le cause che portano allo sviluppo del cancro.
Sappiamo ormai per certo che i tumori sono determinati da eventi genetici, ovvero modificazioni del genoma riconducibili a tre fattori: infezioni virali o agenti infettivi, come il virus del papilloma o dell'epatite, carcinogeni chimici e fisici (il fumo di sigaretta e l'asbesto) e scorretti stili di vita. Ad esempio, il sovrappeso aumenta il rischio di sviluppare tumori e, in particolare, l'obesità è causa ormai scientificamente accertata di cancro.
Alcuni tumori, tuttavia, non sono facilmente spiegabili con nessuna di queste cause. Personalmente sono convinto che alla loro base ci sia un meccanismo più profondo, legato all'evoluzione nel senso darwiniano del termine. Un certo grado di instabilità genetica è intrinseco al processo evolutivo: se il genoma fosse cristallizzato e stabile, la vita sul pianeta non si sarebbe evoluta.
In questo contesto generale, recentemente Cristian Tomasetti e Bert Vogelstein hanno pubblicato su Science un articolo in cui, sulla base della frequenza di cellule staminali e di attività replicativa nei diversi organi, è stata stimata l'incidenza di trasformazione spontanea delle cellule normali in tumorali, in assenza di carcinogeni. Questo lavoro si è prestato ad una lettura provocatoria che ha portato alla

divulgazione di un messaggio fuorviante: “il cancro è una questione di sfortuna, due tumori su tre dipendono dalla cattiva sorte e non dallo stile di vita o dai geni”. Che la sfortuna sia un elemento fondamentale della vita è fuor di dubbio, così come il fatto che ci si possa ammalare anche in assenza di fattori di rischio. Lo studio di Vogelstein e Tomasetti è in realtà interessante ma, come nei confronti di qualsiasi ricerca scientifica, dobbiamo porci domande sui suoi confini e limiti: è fondato sostanzialmente su analisi matematiche e inferenze per organi di cui non abbiamo i dati. Pertanto, le stime che ne conseguono vanno prese con cautela. Inoltre, lo studio ha escluso due patologie ad elevata diffusione, il cancro della mammella e quello della prostata. Questo nulla toglie alla sua qualità, ma di certo “toglie vento alle vele” del messaggio nella forma in cui è stato divulgato.

Quindi, pur senza dimenticare che la fortuna è una variabile esistente e in nessun modo controllabile, dal punto di vista della prevenzione è nostro dovere fare tutto il possibile. Ciò significa innanzitutto seguire stili di vita corretti. In primis - ma non solo - a tavola, dove inizia una buona qualità di vita: una dieta equilibrata, ricca di frutta e verdura fresche, svolge un’azione-chiave nella riduzione del rischio di malattia, perché aiuta l’armonico funzionamento del sistema immunitario. Mi piace sintetizzare i consigli per uno stile di vita corretto nella regola 0-5-30: ogni giorno 0 sigarette, 5 porzioni di frutta e verdura, 30 minuti di attività fisica. Prevenzione inoltre significa evitare, nel possibile, di esporsi ad elementi carcinogeni dell’ambiente, come l’asbesto o i particolati (smog). E significa utilizzare i vaccini disponibili, in attesa che ne arrivino altri.

Perché se nulla si può fare per cambiare la fortuna, la prevenzione è invece nelle mani di ognuno di noi. La lotta al cancro, quindi, passa anche da noi.

Infiammazione e Cancro

9

Regolazione trascrizionale ed epigenetica dell'infiammazione: il contributo della genomica

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Ogni organismo è esposto ad un ampio spettro di stimoli ambientali a cui reagisce costantemente e nella maggior parte dei casi impercettibilmente. Nel caso specifico dei microbi, la capacità di recepire la loro presenza ed organizzare adeguate risposte difensive, è sicuramente una proprietà essenziale per la vita multicellulare sulla Terra. È altrettanto essenziale che ogni risposta venga aggiustata sulla base della natura e dell'intensità dello stimolo in maniera da prevenire reazioni eccessive ad agenti non dannosi così come risposte inadeguate a microbi che potrebbero mettere a repentaglio l'integrità e la sopravvivenza dell'organismo. La rilevanza di questo "tuning" della risposta per la sopravvivenza degli organismi multicellulari è dimostrata dalle proprietà evolutive dei geni che codificano proteine che partecipano alla risposta agli stimoli ambientali: mentre i geni che controllano lo sviluppo e l'organizzazione del piano corporeo sono altamente conservati in specie multicellulari anche molto distanti nell'evoluzione, i geni delle risposte ambientali manifestano un elevato tasso di duplicazioni, perdite e mutazioni nel corso dell'evoluzione. Esempi tipici di questa evolutività sono gli scavenger receptors, i geni codificanti chemokine ed i Toll-like receptors (TLRs) coinvolti nel riconoscimento microbico. Questa notevole propensità all'innovazione (e la conseguente specializzazione nel repertorio dei geni della risposta ambientale) riflette infatti la diversità degli ambienti e dei microbi cui differenti specie sono esposte. A livello dei singoli organismi, la specializzazione nel riconoscimento e nella risposta ai microbi si riflette nella diversi***tà*** dei programmi di espressione genica attivati da stimoli diversi, sebbene altamente relati. Un caso paradigmatico è rappresentato dalla attivazione selettiva dei geni regolati da interferon da parte di un subset specifico dei TLR.

Alla base della risposta infiammatoria agli stimoli sia di natura microbica che non microbica si trova l'attivazione di complessi programmi di espressione genica innescati da recettori di membrana o intracellulari accoppiati a specifiche vie di segnalazione ed infine a fattori trascrizionali. A loro volta i fattori trascrizionali agiscono in maniera combinatoriale su elementi regolatori contenuti nel genoma dei mammiferi e deputati alla attivazione o repressione della trascrizione dei geni adiacenti. L'accessibilità dei milioni di elementi regolatori contenuti nel genoma

dei mammiferi complessi differisce da tipo cellulare a tipo cellulare, riflettendo l'azione di fattori trascrizionali deputati al controllo del differenziamento. Pertanto le risposte infiammatorie a stimoli identici sono influenzate dal contesto (tipo cellulare e tessuto) in cui avvengono.

10

Neuroinflammation: impact on long term recovery after traumatic brain injury

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Il trauma cranico (Traumatic Brain Injury, TBI) è una condizione patologica che interessa milioni di pazienti nel mondo e rappresenta una delle principali cause di morte e disabilità. L'incidenza di TBI, considerato in tutte le sue forme, dalle più lievi alle più gravi, varia nei differenti paesi, riflettendo variazioni locali nella frequenza dell'evento traumatico, ma anche metodologie e criteri di inclusione molto variabili nei diversi studi. I tassi di incidenza registrati variano infatti tra i 60 e i 720 casi per 100.000 abitanti/anno (1).
Nelle ultime decadi, il miglioramento delle cure mediche ha portato ad una riduzione della mortalità che si attesta oggi intorno al 30-40% (2). L'incremento della sopravvivenza si è però tradotto anche in un aumento dei pazienti con disabilità permanenti. Un TBI grave incide negativamente sulla qualità di vita, compromette le precedenti relazioni sociali, l'impiego lavorativo, la vita economica dell'individuo e diminuisce l'aspettativa di vita costituendo dunque un problema socioeconomico estremamente rilevante. I costi stimati totali sono di circa US$ 400.000 per paziente con TBI grave (2). Non stupisce che un TBI grave sia associato a conseguenze disastrose e durature, tuttavia dati recenti indicano che anche TBI (apparentemente) lievi possano andare incontro a disabilità permanenti.
Ad oggi non siamo in grado di predire l'outcome di questi pazienti con sufficiente precisione, questo è attribuibile ad una imperfetta conoscenza della patofisiologia del danno cerebrale post-traumatico. La trasmissione diretta di energia biomeccanica all'encefalo al momento dell'impatto determina una deformazione/lacerazione del tessuto cerebrale con danno immediato (danno primario). A seguito del danno primario iniziano una serie di cascate biochimiche e molecolari (eccitotossicità, produzione di radicali liberi, disfunzione mitocondriale, alterazioni dell'omeostasi del calcio, infiammazione, etc.) che perdurano nel tempo (giorni, mesi e addirittura anni) e hanno un impatto cruciale nel determinare l'esito a lungo termine dei pazienti traumatizzati (3). Comprendere i meccanismi biochimici e molecolari che trasformano un evento acuto biomeccanico in un processo dinamico cronico neurodegenerativo è di fondamentale importanza per sviluppare nuovi approcci terapeutici. Evidenze sperimentali e cliniche suggeriscono che la neuroinfiammazione possa avere un ruolo determinante in numerose conseguenze croniche del TBI. Il TBI induce una risposta infiammatoria acuta e persistente che include la produzio-

ne locale cerebrale di citochine e chemochine, l'attivazione endoteliale, l'attivazione della microglia e il richiamo e la migrazione nel sito di lesione di neutrofili, linfociti e monociti circolanti. La microglia è il maggior contribuente cellulare alla risposta infiammatoria post-TBI. In ambito sperimentale è stato dimostrato che dopo un TBI, le cellule microgliali si attivano precocemente (4) e rimangono cronicamente attive per almeno un anno (5), diffondendosi dal sito di lesione a regioni cerebrali remote. Dati clinici confermano l'importanza in termini di entità e durata temporale della risposta infiammatoria. Studi condotti con tecniche di imaging PET (positron emission tomography, PET) con (^{11}C)PK11195 (che marca la proteina traslocatrice mitocondriale TSPO presente nella microglia attivata) hanno evidenziato una microgliosi cronica (fino ad anni dopo il trauma) nei pazienti TBI. Studi autoptici hanno dimostrato un'attivazione cronica della microglia nel tessuto cerebrale traumatizzato ed un associazione tra entità della risposta infiammatoria e danno della sostanza bianca dimostrando un'associazione in termini spazio-temporali tra microgliosi e fenomeni neurodegenerativi (6, 7). Rimane ancora da chiarire se l'infiammazione abbia un ruolo causale nella propagazione del danno o costituisca piuttosto un epifenomeno dei processi neurodegenerativi in atto.

Il ruolo delle cellule immunitarie dopo danno cerebrale acuto è estremamente complesso, è noto infatti che, in aggiunta alle azioni pro-infiammatorie, "tossiche", microglia e macrofagi sono necessari per la risoluzione del danno/riparazione del tessuto danneggiato. Ad esempio attraverso il rilascio di molecole pro-infiammatorie, come IL1β, TNF-α, proteasi e specie reattive dell'ossigeno, microglia e macrofagi contribuiscono all'esacerbazione del danno tissutale. Viceversa attraverso la regolazione dell'uptake del glutammato, la rimozione di detriti cellulari e la produzione di fattori neurotrofici come *l'insuline-like growth factor-1* (IGF-1), il *glial cell-derived neurotrophic factor* (GDNF) e il *brain-derived neurotrophic factor* (BDNF) contribuiscono a fenomeni di risoluzione del danno e neuroriparazione. Questo duplice ruolo delle cellule immunitarie è altamente influenzato dal microambiente tissutale. I segnali provenienti dall'ambiente extracellulare danneggiato possono indirizzare queste cellule verso funzioni diverse. In vitro, la stimolazione della microglia attraverso ligandi del toll-like receptor (TLR) e IFN-γ induce una polarizzazione di queste cellule verso un fenotipo pro infiammatorio (denominato M1), mentre citochine come IL-4/IL-13 ne favoriscono la differenziazione nel fenotipo pro rigenerativo (denominato M2) (8). I fenotipi M1 e M2 rappresentano i due estremi della polarizzazione della microglia e dei macrofagi, e la situazione in vivo è molto più complessa e caratterizzata da cellule con fenotipi intermedi che possono coesistere nel tessuto leso. Con questi elementi di cautela la valutazione dei markers fenotipici rimane comunque uno strumento molto importante per comprendere quale risposta è prevalente nel tessuto danneggiato. Studi sperimentali hanno dimostrato che un TBI induce una polarizzazione M2 precoce nell'area perilesionale. Tuttavia tale riposta è di breve durata e nell'arco di una settimana il fenotipo dominante diventa quello M1 (9). Ulteriori ricerche, indirizzate a capire i meccanismi alla base della polarizzazione M1/M2, potrebbero avere un utile risvolto terapeutico. La soppressione aspecifica della risposta infiammatoria non sembra essere una strategia terapeutica percor-

ribile, diversi studi dimostrano che inibire la risposta infiammatoria induce un effetto benefico ma transitorio o in alcuni casi addirittura dannoso a tempi cronici (10). Viceversa in ambito sperimentale dati promettenti sono stati recentemente ottenuti con strategie terapeutiche volte non ad inibire ma ad indirizzare la risposta infiammatoria verso un fenotipo benefico.

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11

Inflammation, autoimmunity and tissue damage

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In systemic autoimmune diseases, chronic activation of the immune system is classically linked to the development of tissue damage. Both the cellular and the humoral arms of the immune system promote an amplification loop fuelling continuing inflammation, activation of intrinsic resident cells, and final tissue injury. Whilst the close inter-relationship between aberrant autoimmune reactions and organ damage has long been recognized in prototypical settings such as lupus nephritis and ANCA-associated vasculitides (1, 2), a wide range of evidences now suggest that immune signaling cascades also profoundly influence the pathologic remodeling of a tissue previously viewed as metabolically inert, that is the skeletal system (3).

Bone loss is a common feature of chronic rheumatic diseases. In particular, rheumatoid arthritis (RA) represents the prototype of a chronic inflammatory disease accompanied by rapid bone loss. In addition to systemic osteoporosis, patients with RA develop periarticular osteopenia and joint erosions early in the disease process. Bone loss in RA results from an imbalance between bone resorption and bone formation (4, 5). The marked increase in bone resorption is mediated by bone resorbing osteoclasts (OC) at the pannus-bone interface and in subchondral bone marrow (6). The differentiation and activation of OCs physiologically requires the binding of RANKL to its receptor RANK on OC precursors (7). Increased OC activity in RA is classically believed to reflect the inflammatory burden in joints. Indeed, several pro-inflammatory cytokines present in the inflamed synovium, such as TNF-α, IL-6, IL-1, and IL-17, have been shown to stimulate OC differentiation and activation either directly through activating OCs or indirectly by inducing RANKL production by synovial fibroblast-like cells, T-cells, or bone marrow stromal cells (8, 9). In parallel, recent studies have shown that pro-inflammatory cytokines not only induce bone resorption, but also contribute to bone loss by direct inhibition of osteoblast differentiation (8, 10).

In some subsets of patients with RA, the chronic inflammatory process has a strong autoimmune component, as highlighted by the recognition of typical autoantibodies, such as antibodies to citrullinated proteins (ACPA) and/or rheumatoid factors, and the therapeutic benefit of depleting B-cells (11, 12). Whereas it is well established that inflammation constitutes a major risk factor for bone destruction

in RA, the impact of autoimmunity on bone remodeling is less well perceived. Clinical, immunological and histopathological data indicate that the "B-cell subset" of RA has more severe and rapidly progressive destructive course of the disease. Longitudinal observations have revealed that patients with recent-onset RA develop more bone erosions and more severe osteopenia when ACPA are present as compared with RA patients without ACPA (13, 14). Other serological markers reflective of B-cell activation, including serum concentrations of the B-cell chemoattractant CXCL13 and of IL-21, have been also associated with increased rates of radiographic progression in early RA (15, 16). From a morphological perspective, B-cell dominated lymphoid infiltrates can be recognised in both the synovial membrane and subchondral RA bone marrow in a proportion of RA patients (17, 18), and appear associated with bone erosion, a pro-osteoclastogenic molecular *milieu*, and increased bone-resorbing OCs (19, 20). Such observations have remained casual rather than causal until very recent years, when data have started indicating that B-cells can directly affect the bone by different mechanism. Whilst activated T-cells have been long recognised as key promoters of bone damage in RA joints, recent studies have described RANKL expression by human B-cells (21-23). In particular, switched memory B-cells produce RANKL in quantities exceeding that produced by T-cells upon stimulation, and synovial RA B-cells spontaneously produce RANKL and promote greater osteoclastogenesis than B-cells from healthy controls (23). More intriguingly, RA-associated autoantibodies appear to directly impact on bone remodeling through inflammation-dependent and –independent mechanisms. Indeed, as with any antibody to a self-antigen, ACPA can form immune complexes that can induce inflammation. Immune complexes containing citrullinated fibrinogen or histones have been shown to activate macrophage cytokine production via costimulation of TLR4 and Fcγ receptor (24-26). Furthermore, in vitro experiments have shown that, independent from the formation of immune complexes, purified ACPA can directly activate monocytes by binding to a citrullinated GRP78 cell-surface receptor, driving NF-κB activation and cytokine production (27). The ACPA-mediated increase in TNF-α production by macrophages would thus enhance osteoclastogenesis through classic, inflammation-dependent mechanisms. However, evidence has emerged that autoantibodies might directly stimulate OCs beyond inflammation. OCs and OC precursors indeed express citrullinated vimentin on their surface, and ACPA directed against mutated vimentin and purified from serum of patients with RA can induce OC activation in vitro and bone resorption in vivo after transfer to mice (28). The process of ACPA-induced OC differentiation appears dependent on a combination of intracellular protein citrullination in the OC as well as autocrine production of IL-8 (29). The recent finding that, in an animal model, the administration of ACPA induced trabecular bone loss in the absence of synovial inflammation, and bone loss was inhibited by IL-8 but not TNF blockade (29), reinforces the concept that antibodies may mediate inflammation-independent tissue damage. This data appears very intriguing since ACPA may be locally produced by ectopic synovial lymphoid structures in RA (30). Much work needs to be done to identify more precisely the antigenic targets and the fine characteristics of ACPAs

before definitive conclusion on their direct and causal involvement in human pathology can be drawn. Indeed, not all ACPAs are pathogenic, and characteristics such as avidity and IgG glycosylation seem important in determining the strength of OC activation and the amount of joint destruction (31, 32).
As pathologic bone remodeling is driven by inflammatory stimuli, abating disease activity remains the major goal of any treatment strategy in patients with RA. However, the recent demonstration that B-cells may directly boost OC activity via antibody-independent and -dependent mechanisms provides mechanistic explanation of the increased rates of local and systemic bone loss observed in patients with ACPA-positive RA irrespective of disease duration and activity (33, 34). Direct targeting of autoreactive B-cells and pathogenic autoantibodies may thus represent additional weapons in the war on autoimmune-mediated bone loss.

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Immunoterapia

12

Virus, infiammazione ed epatocarcinoma

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L'epatocarcinoma (HCC) è il più frequente tumore primitivo del fegato (1), responsabile di circa 662,000 decessi l'anno (2). Il più comune fattore di rischio è rappresentato dall' infezione da virus dell'epatite C (HCV) e B (HBV) (3). Altre cause sono l'abuso di alcol, alcune malattie metaboliche ereditarie (emocromatosi e deficit di alfa-1-antitripsina) e la sindrome metabolica che ha, come corrispondente patologia epatica, la steatosi (4).
In corso di un'epatopatia cronica, lo sviluppo di HCC è da considerarsi un processo multistep, noto come epatocarcinogenesi, caratterizzato dall'accumulo progressivo di alterazioni genetiche che conducono verso una trasformazione maligna del parenchima cellulare epatic (5).
Alla base dello sviluppo di epatocarcinoma, oltre ad una eziologia legata ai fattori predisponenti un danno epatico, si aggiungono fattori molecolari; è noto il ruolo centrale della alterata regolazione epigenetica e della flogosi cronica nel processo di epatocarcinogenesi (6).
Con il termine epigenetica si intendono tutti i cambiamenti nei tratti fenotipici di una cellula. Numerosi studi sono stati pubblicati nel tentativo di identificare firme molecolari ("epigenetic patterns") di espressione genica nell'epatocarcinoma (6, 7). Per quanto i meccanismi tra infiammazione ed HCC non siano del tutto chiari, è noto che l'epatocarcinoma rappresenta il tipico esempio di tumore correlato al processo infiammatorio epatico.

Infiammazione cronica in risposta al danno epatico ed alle infezioni virali

Gli epatociti sono costantemente esposti a diversi agenti potenzialmente patogeni e, in condizioni fisiologiche o parafisiologiche, il processo infiammatorio è in grado di contrastare completamente la noxa patogena. Quando il danno epatico risulta essere più esteso e severo, si innescano processi riparativi che esitano, tuttavia, con l'infiltrazione del tessuto epatico da parte delle cellule competenti, quali neutrofili, monociti, macrofagi, eosinofili, cellule dendritiche e linfociti. La tempesta citochinica che ne deriva sembrerebbe sostenere il linkage tra infiammazione e alterazioni geniche, le stesse alterazioni che si osservano negli stadi pre cancerogeni (9, 10).
Infatti, i principali effettori chimici di danno sono rappresentati dai radicali liberi, ROS e RNS e lo stress ossidativo si riflette come danno del DNA cellulare con

conseguente acquisizione di mutazioni che contribuiscono all'epatocarcinogenesi (11).
Lo scenario si complica in corso di infezioni da virus epatotropi e quando il danno epatico è associato ad eccessivo consumo d'alcol e/o in caso di steatosi epatica (12, 13). Nel caso dell'infezione da HBV, il DNA virale esercita il suo potenziale cancerogeno tramite l'integrazione dello stesso a carico di oncogeni o di oncosoppressori (p53, Rb, cyclin D1 e p16) inducendo pertanto processi di instabilità cromosomica, quali delezioni, duplicazioni e traslocazioni di regioni cromosomiche. Per esempio l'espressione del prodotto genico HBx provoca una mancata regolazione trascrizionale inducendo un'aumentata proliferazione cellulare e una progressione del ciclo cellulare; inoltre HBx esercita ruolo di transattivatore a livello di oncogeni ad esempio, come c-myc, c-Fos e c-Jun (14).
Il meccanismo di epatocarcinogenesi correlato all'infezione da HCV non è ancora stato chiarito, ma è stato dimostrato che il virus replica all'interno delle cellule neoplastiche; non è invece stata dimostrata la presenza di oncogeni nel genoma del virus né la sua capacità di integrarsi nel genoma delle cellule epatiche. Questo suggerisce che l'HCC si sviluppa attraverso l'induzione di un danno cronico infiammatorio (8).

Stimolo citochinico e sviluppo epatocarcinoma

La steatosi e la fibrosi, di qualsiasi eziologica, promuovono importanti cambiamenti del pattern citochinico e correlano direttamente con la progressione di malattia. Nello specifico si rilevano alterazioni a carico dei livelli di TNFα, IL6, IL1α, IL1βα and IL10 e TGFβ (transforming growth factor beta). Nella fase precancerosa l'espressione citochinica risulta sostanzialmente sovrapponibile al microambiente del tessuto cirrotico, suggerendo, pertanto, un ruolo centrale delle citochine nel processo di carcinogenesi. Come emerge da alcuni studi, ad esempio, caratteristica è la riduzione dei valori di TNFα e IL6 nel processo di epatocarcinogenesi (15, 16).

Attivazione del NF-κB e STAT3 pathway ed epatocarcinogenesi

Nel processo di carcinogenesi, anche in considerazione della conseguente tempesta citochinica, si riscontrano importanti alterazioni a carico di pathway implicati nella proliferazione cellulare, durata replicativa, processo di apoptosi, differenziamento e integrità genomica. Alterazioni a carico delle NF-κB e STAT3 pathways sembrerebbero avere un ruolo pro-carcinogeno (17).

Meccanismi epigenetici ed epatocarcinogenesi

Lo sviluppo di un fenotipo tumorale si esprime a seguito all'accumulo di aberrazioni genetiche diverse. Le alterazioni epigenetiche a cui si può imputare l'epatocarcinogenesi coincidono con una aberrante metilazione dei geni promotori e con una disregolazione della espressione dei microRNA (miRNA). Ad esempio gli

eventi di metilazione (ipo e ipermetilazione) possono promuovere silenziamento genico (18). Ad oggi numerosi studi riportano l'analisi dei profili di espressione dei microRNA nell'epatocarcinoma umano ed in modelli sperimentali di HCC. L'up-regolation di microRNA-18 (miR-18), miR-21, miR-221, miR-222 and miR-224, cosi come la downregulation of miR-122, miR-125, miR-130a, miR-150, miR-199 and miR-200 sono riportati nell'epatocarcinoma suggerendone il coinvolgimento nel processo di epatocarcinogenesi (19).

Conclusioni

Nonostante gli importanti progressi, non è del tutto chiaro il livello di interazione tra infiammazione ed eventi epigenetici. Diverse rimangono le questioni aperte: possono le citochine infiammatorie fungere da trigger per gli eventi epigenetici? Oppure può la deregolazione epigenetica contribuire a sostenere il processo flogistico?
Pertanto la caratterizzazione di meccanismi genetici, epigenetici ed infiammatori alla base dello sviluppo dell'HCC al fine diagnostico e prognostico rimane una prospettiva futura di ricerca.

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13

Understanding the Rationale for Immunotherapy of Tumors

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The first demonstration of the immunogenicity of tumors dates back to the '50s, when researchers demonstrated that mice bearing methylcholanthrene-induced tumors resected by surgery, proved resistant to a second graft of the same tumor (1, 2). Of note, no protection was observed against other syngeneic tumors, indicating that the tumor specific antigens carried by individual tumors were different. Later experiments showed that tumors induced by ultraviolet radiation or virus-induced tumors (*e.g.* tumors induced by polyoma and SV40 viruses) also expressed specific antigens causing rejection (3, 4). T-lymphocytes isolated from the spleen of immune mice were shown to be the final effectors of the antitumor responses. Altogether, these observations led to the formulation of the immune surveillance hypothesis, as proposed by Burnet and Thomas in 1957 (5, 6).
The observations in carcinogen- and virus-induced tumors were however not confirmed in spontaneous mouse tumors. Hewitt reported that irradiated tumor cells were unable to generate any immune protection against grafts of the same tumor (7), thus suggesting that tumor antigens were either viral antigens or artifacts induced on rodent tumors by "non-physiologic" amounts of carcinogens, and therefore unlikely to match with conditions causing human tumors. These experiments were challenged by Boon and colleagues, who showed the generation of immunogenic tumors from non-immunogenic tumors (tum^- variants) by *in vitro* treatment with chemical mutagens (8, 9), and by identifying human tumor antigens recognized by specific cytotoxic T-cells in the early '90s (10).
Over the last 25 years accumulating evidence at molecular and cellular levels have demonstrated the key role played by the immune system in the control of tumor growth. Here, I will give a personal point of view about the most compelling evidence of the immune-mediated tumor growth control.
In 2001 Schreiber and colleagues evaluated the incidence of methylcholantrene-induced sarcomas in wild type and immunodeficient mice, particularly mice lacking T-cells ($RAG^{-/-}$ mice) or mice lacking IFN-g signaling (11). Immunodeficient mice showed an increased incidence of carcinogen-induced sarcomas as compared to wild type mice. Furthermore, immunodeficient mice developed tumors more rapidly than wild type controls, thus indicating an active role of T-cells and IFN-g signaling in cancer immune surveillance. Moreover, by monitoring these mice for

24 months, they also showed after necropsy that these mice were more prone to develop spontaneous tumors histologically similar to those developing in elderly subjects (*i.e.* lung, colon and breast carcinomas). This observation led to a revised cancer immune surveillance hypothesis based upon three phases (*The three Es of cancer immunoediting*):

1) *Elimination* phase occurring at early times when transformed cells start growing damaging neighboring health tissues and leading to a prompt immune response with tumor cell killing;
2) *Equilibrium* phase in which cancer cells enter a dormancy state. In this phase there is a balance between tumor cell killing and proliferation. Of note, in this phase there is a continuous antigenic editing of tumor cells with the selection of tumor cells endowed with poor immunogenicity (tumor cells mostly expressing weak antigens). This phase may last several years (10-20 years), a condition explaining the long phase of tumor progression of certain human tumors (*e.g.* breast and colon cancers);
3) *Escape* phase in which tumors adopt distinct mechanisms to avoid immune system elimination, thus dampening the antitumor immune response. In this phase the tumors are clinically evident and based upon the high degree of molecular and cellular heterogeneity they invade neighboring and distant tissues to give rise to metastases (12, 13).

Compelling evidence on the pivotal role played by the immune system in controlling tumor growth in humans came from the observations of patients undergoing kidney, heart and liver transplantation and chronically treated with immune suppressive drugs to avoid the transplant rejection. A strong increase of the development of certain tumor types has been observed in these patients. Transplant patients develop more frequently skin carcinomas, tumors of the anogenital region, melanoma, lymphomas and sarcomas (14). In particular, squamous-cell and basal-cell carcinomas account for more than 90 percent of all skin cancers in transplant recipients (14). The incidence of these carcinomas increases with the duration of immunosuppressive therapy (14). Most immune suppressive drugs used in transplant patients blunt adaptive immune cells (*i.e.* T-cells), which are the ultimate effectors of the antitumor immune responses.

The number and the subset of T cells, particularly tumor-infiltrating lymphocytes (TILs), have recently provided an additional line of evidence linking tumors to immune surveillance in humans. Indeed, in ovarian cancer samples the number of tumor-infiltrating $CD8^+$ T-cells was shown to correlate with an improved overall survival and with a complete response to therapy, considering 174 and 74 stage III or IV ovarian cancer patients, respectively (15). In colon cancer, it was shown an increased overall and disease-free survival in patients with a high density of $CD8^+CD45RO^+$ T-cells, indicating that in addition to the number of $CD8^+$ T-cells it is also relevant the status of differentiation of these tumor-infiltrating $CD8^+$ T-cells (memory T-cells) (16).

This observation begs the question about the target recognized by these tumor-infiltrating $CD8^+$ T-cells. As anticipated below, in 1991 Boon and colleagues described the molecular structure of the first human tumor antigen, *i.e.*

MAGE-A1 (Melanoma Antigen E-1), recognized by cytotoxic ($CD8^+$) T-cells on the surface of autologous human melanoma cells as a complex between a given HLA-I molecule and a tumor-derived peptide (17). Specifically, they reported that $CD8^+$ T-cells recognized a nonapeptide generated by the intracellular processing of the MAGE-A1 protein in association with HLA-A1 molecules (18), laying the foundations for the molecular comprehension of the recognition of tumor-associated antigens by T-cells (10). Indeed, several other tumor antigens recognized by $CD8^+$ T-cells were identified as restricted to different HLA-I alleles (19). Moreover, peptides restricted by HLA-II alleles and recognized by specific $CD4^+$ T-cells were also identified (20, 21). Tumor antigens can be classified according to their expression, the mechanism of generation, etc. Activated tumor antigens comprise the cancer-germline genes, which include several families of tumor antigens, such as MAGEs, BAGEs, GAGEs, NY-ESO-1/Lage antigens that are silent in normal tissues with the exception of male germline cells and trophoblastic cells (19). Cancer-germline genes are activated by DNA demethylation and are shared by different tumor histotypes, *e.g.* melanoma, lung, breast, sarcoma, etc. (Table 1). Differentiation antigens include antigens expressed only in the tumor cells and in the normal tissue of origin. MelanA/MART-1, gp100, tyrosinase and other antigens represent differentiation antigens expressed by melanoma cells and by non-transformed melanocytes (22, 19). Broadly speaking, antigens belonging to the cancer-germline and differentiation genes/antigens are classified as "self antigens"; although, this term is misleading when used to describe the cancer-germline antigens (23). However, a high degree of T-cell tolerance against these antigens is common. Unique tumor antigens arise from non-synonymous mutations occurring in driver or passenger genes (24, 25). These antigens are considered stronger tumor antigens, since mutations generate "non-self" epitopes recognized by high avidity T-cells. Based on this, tolerance against these antigens is normally absent or poorly present. Since three years the unique antigens or neoantigens are under active investigation as T-cells against these antigens have been frequently found increased in the blood of melanoma and lung cancer patients undergoing immune checkpoint blockade therapy (anti-CTLA-4 and anti-PD-1 mAbs) (26).

Tab. 1 - Expression (%) of Cancer-germline genes by different tumor histotypes.

Tumor histotype	MAGE-A1 (%)	MAGE-A3 (%)	BAGE (%)	GAGE (%)
Melanoma	46	70	20	25
Lung (NSCLC)	46	47	6	19
Sarcoma	7	13	0	17
Head & Neck	31	51	6	26
Ovarian cancer	28	17	15	31
Breast cancer	20	13	10	9
Colorectal cancer	0	17	0	0
Kidney cancer	5	0	0	0

Percentages of expression in different tumor histotypes arise form the studies reported in refs (58), (59), (60), (61), (62).

Until the 2000s, oncologists considered the cell-intrinsic properties regulating cell proliferation, apoptosis, senescence, angiogenesis, invasion and metastasis as the main hallmarks of cancer cells (27). After the 2000s, due to the growing evidence of cell-extrinsic mechanisms regulating some tumor processes, both tumor-promoting inflammation and the immune escape were added as emergent hallmarks of cancer cells (28). A strong association between inflammation and cancer has been suggested by clinical and epidemiological studies (29, 30). Indeed, inflammatory molecules, such as cytokines (IL-6, IL-23, TNFa, IL-1b, etc.) and some transcription factors (NF-kB, STAT-3) (31),(32) can provide growth signals promoting the neoplastic transformation of normal cells, the proliferation of malignant cells, as well as tumor metastasis (33). Recently, it has been demonstrated that the transient activation of the non-receptor tyrosine kinase v-Src causes an epigenetic switch responsible for neoplastic transformation of mammary non-transformed cells by linking NF-kB, IL-6, Lin28b, Let7 micro-RNA and STAT-3. Transient induction of Src activates NF-kB, which induces the expression of Lin28b, which in turn reduces the levels of let-7 micro-RNA. The inhibition of Let-7 results in marked expression of IL-6. Finally, IL-6 mediates the activation of the STAT3 transcription factor that is necessary for transformation, and further activates NF-kB, thus perpetuating the positive loop initiated by the first wave of IL-6 release (34). The demonstration of the link between inflammation and cancer has opened new avenues for a better understanding of the carcinogenetic process and new possible targets for therapeutic purposes.

Over the last fifteen years a large variety of factors released by tumors and capable of inhibiting antitumor immune responses have been molecularly characterized (35). Among these factors, the activation of metabolic pathways involving the production of toxic catabolites (36, 37) seems to play a critical role in promoting immune suppressive networks. Of note, these mechanisms may be in part responsible for the limited spontaneous and vaccine-induced antitumor immune responses in cancer patients (38, 39).

Immune escape mechanisms can act both on the cells governing the adaptive (dendritic cells, $CD8^+$ and $CD4^+$ T-cells) and the innate (dendritic cells, macrophages, NK and NKT cells) antitumor immune responses (40). Van der Bruggen and colleagues (41) have shown that non-functional CTL clones and anergic TILs do not co-localize the T Cell Receptor (TCR) and CD8 at the cell surface. This is due to the tumor over-expression of galectin-3, a beta-galactoside-binding protein that block TCR mobility on the cell membrane. Moreover, immune cells endowed with tumor antigen-specific immune suppressive function, *i.e.* T regulatory cells and Myeloid-Derived Suppressor Cells (MDSCs), or with pro-angiogenic properties ($CD11b^+Gr1^+$ immature myeloid cells) (42) have frequently been found increased in tumor-bearing mice and cancer patients (43, 44). Moreover, it is now well established that tumor-associated macrophages (TAMs) are endowed with pro-tumor properties (45). Indeed, TAMs often produce and release anti-inflammatory cytokines, such as IL-10, IL-13, nitric oxide (NO) and other molecules suppressing antitumor T cells (46-48).

With regard to DCs, several cytokines, growth factors, chemokines and small

molecules (IL-10, VEGF, M-CSF, SDF-1, LXA4) have been demonstrated to interfere with the generation and/or function of DCs (49, 50).
Our group has recently identified some products of cholesterol metabolism, namely oxysterols, which dampen the antitumor immune responses through Liver X Receptor (LXR)-dependent and -independent mechanisms (51). We have demonstrated that in some tumors the generation of an immune suppressive microenvironment promoting tumor progression is induced by LXR ligands/oxysterols (51). In particular, tumor-derived LXR ligands bind and activate the nuclear receptor LXRa on maturing dendritic cells (DCs), thereby inhibiting the functional up-regulation of the chemokine receptor CCR7 on their surface and inhibiting their migration to secondary lymphoid organs where DCs activate antigen-specific naive T- and B-cells (52) (Fig. 1A). Moreover, we have demonstrated that tumor-derived oxysterols attract neutrophils endowed with pro-tumor functions within the tumor microenvironment (53). These neutrophils suppress antigen-specific T-cells and promote neo-angiogenesis (Fig. 1B) (53). Of note, the pharmacological or genetic inactivation of oxysterols restores the antitumor immune responses leading to tumor growth control or rejection (54).
Given these premises it is not surprising that immunotherapy of cancer has recently reached clinical success. Indeed, the antitumor responses obtained with antibodies blocking immune checkpoint molecules, such as CTLA-4 and PD-1, which are expressed on activated T-cells (55) has formally elevated cancer immunotherapy to the Olympus of neoplastic treatments (56).

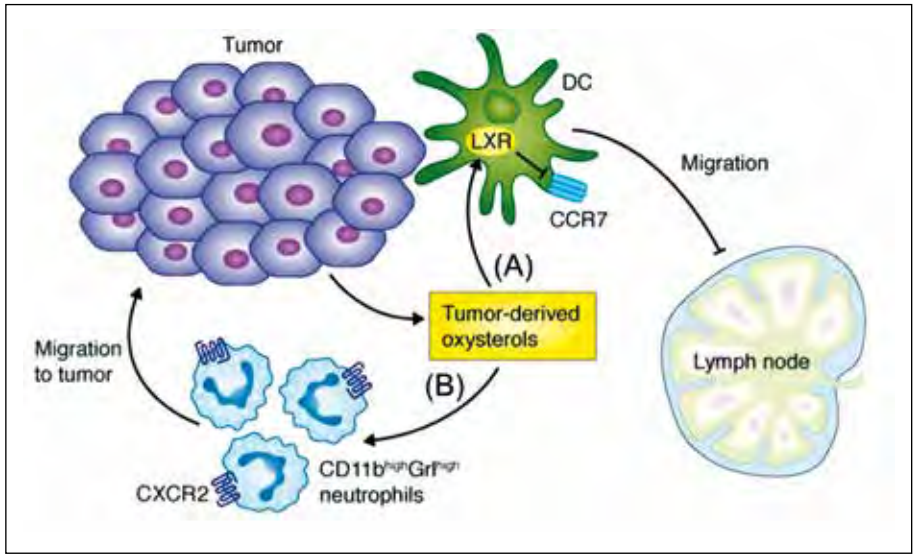

Fig. 1 (A) - Tumor-derived oxysterols inhibit the expression of CCR7 on tumor-infiltrating DC, thus blocking DC migration to secondary lymphoid organs, in an LXR-dependent manner. **(B)** Tumor-derived oxysterols recruit pro-tumor neutrophils within the tumor microenvironment in an LXR-independent, CXCR2-dependent manner. These LXR/oxysterol-based tumor immune evasion mechanisms dampen antitumor immune responses ultimately promoting tumor progression.

Adapted from York A.G. and S.J. Bensinger J. Exp. Med. 2013; 210 No. 9: 1653-56 (63).

Of note, the clinical use of the anti-CTLA-4 antibody Ipilimumab has doubled the overall survival of metastatic melanoma patients. Based on these results, in 2011 the Food and Drug Administration (FDA) has approved Ipilimumab for the treatment of metastatic melanoma patients (57). Recently, the anti-PD-1 antibodies Nivolumab and Pembrolizumab have increased the overall survival of patients affected by metastatic melanoma and lung cancer (non-small-cell lung cancer) and have received the FDA approval for the treatment of these tumor entities (56).

Concluding remarks

During the last 20-25 years the existence of tumor-mediated immune surveillance has been demonstrated at cellular and molecular levels. This has led to the generation of new treatments that are revolutionizing the field of oncology. In the near future, we envisage that immunotherapy will represent a consolidated and effective treatment for patients affected by tumors of different histological types.

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14

Protein aggregation and pathways of toxicity

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Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases of humans and many animal species caused by prions. The main constituent of prions is PrPSc, an aggregated moiety of the host-derived membrane glycolipoprotein PrPC. Prions were found to encipher many phenotypic, genetically stable TSE variants. The latter is very surprising, since PrPC is encoded by the host genome and all prion strains share the same amino acid sequence. Here I will review what is known about the infectivity, the neurotoxicity, and the neuroinvasiveness of prions. Also, I will explain why I regard the prion strain question as a fascinating challenge – with implications that go well beyond prion science. Finally, I will report some recent results obtained in my laboratory, which is attempting to address the strain question and some other basic issues of prion biology with a "systems" approach that utilizes organic chemistry, photophysics, proteomics, and mouse transgenesis.

Cell Biology of Prions and Prionoids: A Status Report

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PMDs

Alterations in the secondary structure of proteins can lead to the formation of abnormally folded conformers. On reaching critical thresholds, or under the influence of poorly understood triggers, these can seed the formation of ordered aggregates and ultimately lead to PMDs (1, 2). These disorders can occur in many different organs; for example, in the form of amyloidoses in the liver, the spleen, or the peripheral nervous system. This review focuses on PMDs affecting the central nervous system, which include Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and prion diseases. The primary structures of misfolded proteins, and the clinical consequences of their misfolding, vary between these diseases, yet all of these entities share common features including the loss of neurons, abnormalities of synaptic function, and, most conspicuously, the deposition of extracellular or intracellular protein aggregates.

Most evidence indicates a model of disease progression common to all PMDs. Abnormally folded, or partially unfolded, conformers of a disease-associated protein interact with each other to form cross-beta spines (3) that assemble into a 'nucleus', an ordered aggregate possessing the ability to self-propagate. Therefore, such nuclei were appropriately called 'propagons' (4) even if the propagation process is incompletely understood. Propagons may accrue additional monomeric protein from their environment, possibly by exploiting transient semi-unfolded states. Since accretion typically occurs along a single axis, its product is not a 3D crystal but rather filamentous structures sometimes called protofilaments. Multiple protofilaments can interact with each other and form higher-order fibrillary aggregates, which can be visualized by microscopy (Figure 1). Fibrils can also fragment and liberate additional seeds, which possess templating (and therefore self-perpetuating) activity of their own. Theoretical considerations (5) indicate that the frangibility (i.e., the intrinsic propensity of fibrils to fragment into smaller units) is a crucial determinant of their self-propagation. A fibril that never breaks would be relatively harmless because it would be unable to maintain the disease-generating process. Substances that reduce aggregate frangibility are therapeutic (6, 7). Note, however, that this model of cyclical propagon amplification does not explain the toxicity associated with the deposition of protein aggregates. Indeed, it is compatible with the existence of 'functional amyloids', which may occur physiologically and exert beneficial biological functions (8).

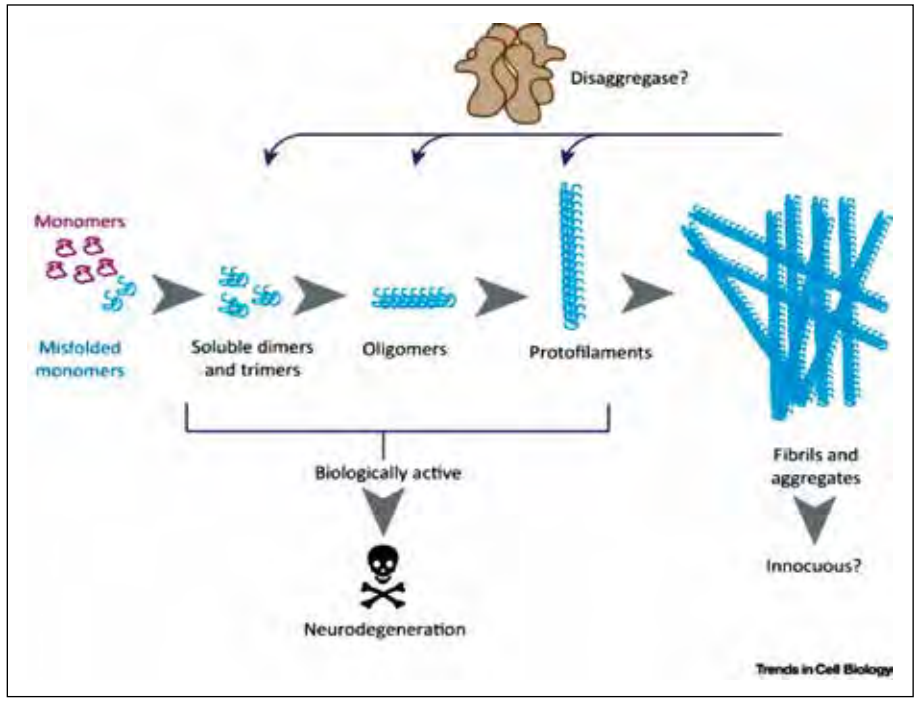

Fig. 1 - The Life Cycle of Protein Aggregates in Protein Misfolding Disorders (PMDs). The partial unfolding of proteins can result in the formation of abnormally folded soluble dimers and trimers. These can associate with each other or with additional monomers resulting in larger species. Further assembly results in protofilaments, which interact with each other to generate fibrils and larger structures including plaques and tangles. The precise composition of the 'propagon' (i.e., the minimal self-replicating species) is unknown. Likewise, it is unknown whether the toxic species is identical with the propagon. It is often said that large fibrils may be harmless, yet large aggregates exist in equilibrium with oligomeric species and the latter can spawn from the former. Fragmentation of larger aggregates can be spontaneous or catalyzed by disaggregases and liberates further oligomeric species, which can seed further aggregation.

Among the best-characterized PMDs are prion diseases, which result from misfolding and aggregation of cellular prion protein (PrP^{C}) into highly ordered, beta sheet-rich aggregates. By our definition (9), the 'prion' is the agent that causes prion diseases (also called transmissible spongiform encephalopathies (TSEs)). Crucially, this definition does not assign a specific physical entity to the word 'prion'. Instead, the prion is simply a propagon associated with TSEs. While there is little doubt that the prion comprises primarily a misfolded (and perhaps post-translationally modified) version of PrP^{C}, it can assume various forms ranging from large, insoluble fibrillary aggregates and plaques that resist proteolytic digestion (and are termed PrP^{Sc}) to small, soluble, protease-sensitive oligomers comprising only a few molecules. Because of their higher molar ratio, these oligomers may possess higher biological activity relative to their weight, and >90% of prion infectivity from brain homogenates is typically protease sensitive. Conversely, many methods result in the conversion of recombinant PrP into a protease-resistant form, but this conversion does not necessarily give rise to propagons. These

qualifications are essential to understanding prion biology and contradict the common misconception of prions being identical with protease-resistant PrP (10-12). Because beta sheet-rich propagons occur in most PMDs, some authors consider all of these diseases to be caused by 'prions'. In our view this is a misconception, since most PMDs are not infectious yet infectivity is a defining trait of prions. TSEs are transmissible diseases whose causative agent has long been thought to be a virus, and prions share many biological properties with small viruses. Far from being an exceptional incident, the transmissibility of TSEs has caused large epidemics such as kuru in Papua New Guinea and, more recently, bovine spongiform encephalopathy. Prion contamination of pituitary extracts has caused one of the largest iatrogenic disasters recorded in medical history, with hundreds of deaths among young adults and thousands more being at risk of the developing the disease. Because no other PMD was found to be transmissible between humans, the former should not be put into the same category as TSEs, even if the underlying molecular pathogenesis displays striking similarities. We have proposed that PMD proteins capable of cell-to-cell propagation within individuals be denoted 'prionoids' (2, 13). In future studies, some prionoids may be found to be transmissible between individuals, thereby causing infectious diseases. If that is the case, it will be appropriate to upgrade their status to *bona fide* prions with all of the unavoidable consequences related to biosafety classification and precautionary measures aimed at preventing their spread.

Oligomers: Seeds of Self-Sustained Propagation in PMDs

Prion Protein

Prion diseases can be acquired, genetic, or sporadic (14). Iatrogenic prion transmission (e.g., through contaminated surgical equipment) continues to occur because prions resist many of the conventional methods of sterilization and prion-detection procedures are disappointingly insensitive. Current evidence suggests that PrP^C can fold into various self-propagating conformations, each one of which can propagate its own distinctive histopathological and biochemical signature (10). Because these features are closely reminiscent of viral strains, they have been termed prion strains (15). Distinct conformers of other PMD proteins, including Aβ, tau, and synuclein, have also been said to give rise to 'strains' although most of the latter proteins have not been shown to be infective (16, 17).

Infectious conformers of PrP were originally described to be detergent insoluble and protease resistant (18), yet it is now clear that protease resistance is not an obligate feature of prion infectivity. Infectious PrP oligomers are thought to grow by incorporating further monomers and may eventually generate protofibrils and large amyloid deposits. Oligomeric species of PrP can be assembled *in vitro* rather easily and some of these can exhibit toxicity when added to neuronal cell cultures. However, it proved difficult to confer infectivity to such synthetic oligomers, at least not to the titers typical of *bona fide* prions (19). These studies imply that prion replication and prion toxicity are two distinct biological phenomena. Many different types of PrP aggregates can be toxic even if they are not self-replicating,

whereas *bona fide* prions can accumulate in the form of fibrils and plaques and yet not elicit toxicity (e.g., when the target cells lack PrP^C) (20).

Aβ and Tau

AD, the most prevalent PMD, is characterized by the deposition of extracellular Aβ-containing amyloid plaques and intraneuronal aggregates of hyperphosphorylated tau protein in form of neurofibrillary tangles (NFTs) and neuropil threads (NTs) (21). Much evidence implicates soluble oligomeric species of Aβ in the pathogenesis of dementia(22). As with prions, it seems plausible that small oligomers, by virtue of their higher molarity at equivalent weights, possess stronger biological activities. Aβ are proteolytic products of amyloid precursor protein (APP), which is processed into amyloidogenic fragments by the β and γ secretases. Many different peptides (126) are generated *in vivo*, of which $Aβ_{1–42}$ has the highest propensity to aggregate (23). Mice expressing an APP transgene that bears the 'Swedish' mutation (K670N/M671L) produce elevated levels of $Aβ_{1-42}$ peptide and suffer cognitive impairment (24, 25). Mutations in the tau gene cause neurodegeneration with NFTs, thereby providing genetic evidence for the causal role of tau aggregation in disease (26). However, the mechanistic relationship between deposition of Aβ and tau hyperphosphorylation remains unclear (27) and there is still much debate on their relative contributions to AD pathogenesis.

Aβ also interacts with cellular PrP^C through its amino terminus (the 'flexible tail' (FT)) and this interaction seems to trigger the activation of the intracellular tyrosine kinase c-Fyn, which, conversely, is upregulated in AD (28). How this relates to Aβ toxicity, however, is controversial. *Prnp*-ablated mice were reported to resist the toxicity of Aβ oligomers (29), yet a plethora of subsequent studies 30, 31, 32 and 33 have questioned these findings.

Might AD be a transmissible entity similar to prion diseases? Whereas early studies of AD transmission to primates were inconclusive (34), injection of Aβ accelerates the deposition of endogenous Aβ in transgenic mice overexpressing APP (35-38). These phenomena are different from those occurring in prion transmission since they occur only in recipients that overexpress Aβ and are therefore predisposed to the disease. Conversely, overexpression of Aβ seems to reproduce features that have long puzzled prion scientists, such as the formation of strains, which may correspond to distinct conformational states of aggregates (39, 40-42) and are preserved even after transmission to recipients (43,). If Aβ pathologies were found to be transmissible to wild-type hosts, we contend that Aβ would need to be subjected to precautionary measures similarly to *bona fide* prions (45).

Alpha Synuclein (α-Syn)

PD is a neurodegenerative disorder characterized by the accumulation of aggregates called Lewy bodies in the cytoplasm of dopaminergic neurons in the substantia nigra. Over time, this produces progressive toxicity and neurodegeneration manifesting as movement disorders. The Lewy body principally comprises α-Syn. Although some studies have suggested that α-Syn forms a helically folded tetramer, most studies describe α-Syn as an intrinsically unstructured monomer

(46-48). Mutations in the*ASYN* gene encoding α-Syn are linked to familial PD and overexpression of wild type α-Syn is sufficient to cause PD (49). Cytoplasmic levels of α-Syn appear to increase with age (50), yet the factors that trigger PD in specific cohorts of individuals are unknown.
Despite their conspicuous presence in PD neurons, Lewy bodies may not represent the most toxic α-Syn species. If each aggregate is a toxic unit, stoichiometry will dictate that small oligomers be more toxic than large fibrils of equivalent weight (51, 52). In landmark experiments, aggregated α-Syn was found to induce PD-like disease in wild-type mice and rats (53, 54). Because serial transmission of synucleinopathies across several individual hosts was not reported, it remains unclear whether they can propagate as a truly infectious disease.

Huntingtin
HD is characterized by the expansion of a CAG trinucleotide repeat (encoding glutamine) within the *HTT* gene that encodes huntingtin. This results in the accumulation of aggregates bearing polyQ repeats. Disease occurs above a threshold of 35-40 repeats and the age of onset of HD is inversely correlated with the length of the polyQ stretch (55). The clinical manifestation of HD is characterized by alterations in motor functions deriving from damage to the basal ganglia of the brain, but eventually severe cognitive impairment arises from a broad effect on the brain and from widespread cortical atrophy (41, 42).
The mechanism of neurodegeneration in HD and related trinucleotide expansion repeat diseases (such as spinocerebellar ataxias) remains unclear. Atomic force microscopy of the brains of mice overexpressing mutant huntingtin revealed huntingtin oligomers of 20-40 nm in diameter (56, 57) similar to those observed with *A*β and α-Syn (58). Soluble huntingtin aggregates have been reported to be the crucial toxic species, rather than the large aggregates (59), which may represent a protective reaction of cells (60, 61). The toxic species can interact with, and inactivate, transcription factors (62) and possibly also chaperones and proteasome components.

Oligomer Generation
The studies cited above suggest that oligomeric species are the key pathogenic drivers in various PMDs, but where do all the oligomers come from? Fibrils may break spontaneously once they reach a critical length, producing an equilibrium between the growth and fragmentation of aggregates. However, studies in yeast have identified Hsp104 disaggregase, a sophisticated machinery that can dissolve cytosolic aggregates including the yeast prion ψ (63-65). As expected, overexpression of Hsp104 can cure yeast from ψ prions but yeast cells lacking Hsp104 display the 'psi-no-more' phenotype, which renders them resistant to prion infection (66), showing that disaggregation is essential to prion replication. This may seem counterintuitive, yet a fibril that never breaks cannot be infectious, since its fragmentation is equivalent to a replicative event in which many prions arise from a single one (5).
Does a mammalian disaggregase system exist? In the cytosol of mammalian cells, a chaperone triad of Hsp110/40/70 has been proposed to act as a minimal protein

disaggregase but seems unable to match the functionality of Hsp104 (67, 68). Recently, a HSP70 chaperone complex comprising J proteins and HSP110 has been proposed to function as a potent mammalian disaggregase, facilitating the unraveling of protein aggregates of varying sizes (69). Further studies are needed to determine whether this is indeed the elusive mammalian disaggregase complex. PrP^{C} enters the secretory pathway cotranslationally, where it acquires a glycolipid anchor (70). Hence PrP^{Sc} prions exist primarily in the organellar and extracellular space, raising the question of whether prion propagation relies on disaggregases in these compartments. While there is no physical evidence for the latter, there are indirect hints that such a system may yet be discovered. Consider this: the creation or amplification of prions *in vitro* from purified components is extremely inefficient (71) and/or requires extremely harsh, unphysiological conditions (72). By contrast, neuroblastoma cells (73) and organotypic slice cultures (74) can amplify prions by several orders of magnitude at room temperature, in the absence of chaotropes within weeks. The identification of the cellular machinery that enables such amplification is likely to offer inroads into the therapy of protein aggregation diseases.

Cell-to-Cell Spread

Most protein aggregation diseases are associated with ageing, raising the question of whether ageing leads to progressive loss of the capability to deal with misfolded proteins. However, recent evidence suggests that aggregation of disparate proteins and the associated damage seem to spread from one brain area (or even from extraneural sites) ultimately leading to progressive, generalized disease. Transmission from cell to cell involves a donor cell releasing propagons and an acceptor that is competent for maintaining propagation.

Exosomes: Are They or Are They Not?

Toxic oligomers may be packaged by cells into exosomes, which are thought to act as Trojan horses carrying toxic seeds and transfer these to acceptor cells (Figure 2). Exosomes are released from cells when multivesicular bodies (MVBs) fuse with the plasma membrane of the cell and carry constituents of both the cytosolic and endocytic compartments. Both PrP^{C} *and* PrP^{Sc} *are associated with MVBs, and unsurprisingly also with exosomes* (75). However, it is unclear whether exosomes are the main vehicle by which prions move from one cell to the next. Conversely, PrP^{Sc} *might alter endosomal dynamics, promoting exosome secretion. Prionoids may also use exosomes to move out of cells, as suggested by the presence of exosomal components such as Alix in A*β plaques from the brains of AD patients and Aβ-overexpressing mice (76). Intracellular Aβ may be packaged into MVBs, incorporated into exosomes, and released from cells in a 'packaged' format (76, 77). Both APP and its C-terminal fragments are incorporated into exosomes, suggesting that cleavage of Aβ can also occur in these compartments. However, only a minor fraction of Aβ is found associated with exosomes extracellularly, which questions the pathophysiological relevance of the exosome pathway as a route of Aβ spread (78).

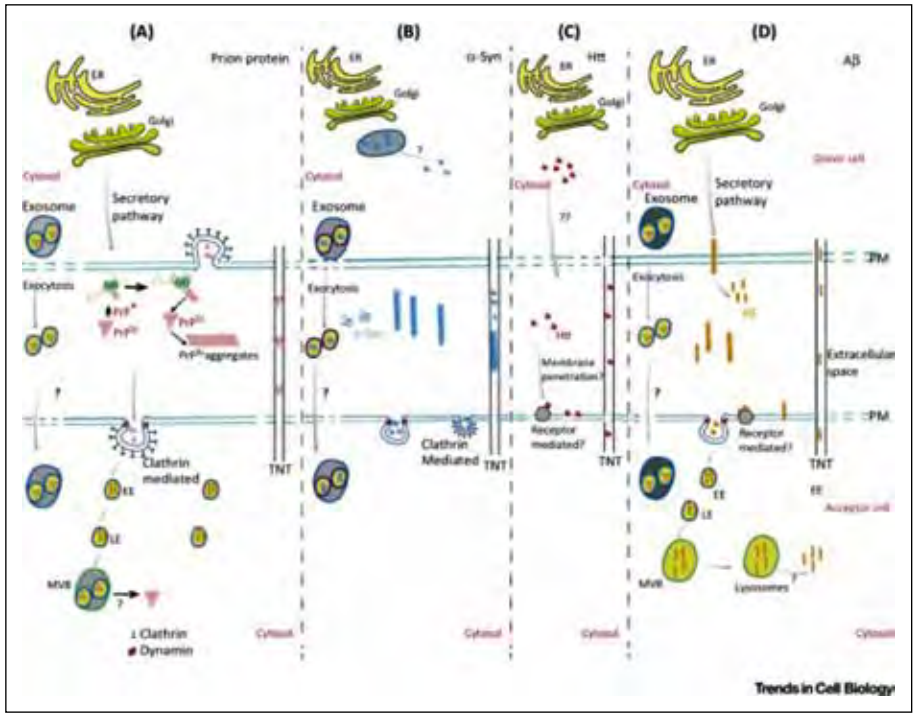

Fig. 2 - Cell-to-Cell Spread of Prions and Prionoids. The upper and lower panels of the figure depict cells that donate and accept propagons, respectively. Cells are separated by the extracellular space. The prion protein PrP^C matures along the classical secretory pathway and reaches the plasma membrane where it is tethered by a glycolipid anchor protein. On encountering a prion propagon, PrP^C can be incorporated into PrP^{Sc}, which was originally defined as a proteinase K-resistant isoform of PrP but the term is often used synonymously with the propagon. The conversion of PrP^C to PrP^{Sc} is thought to occur within the endocytic compartment. Propagon-containing multivesicular bodies may be exocytosed and taken up by acceptor cells. Tunneling nanotubes (TNTs) have also been proposed to transport PrP^{Sc} between cells (A). Alpha synuclein (α-Syn) is a cytosolic protein that, on encountering misfolded isoforms, may undergo conformational changes resulting in the formation of oligomers. α-Syn is associated with vesicles and is thought to exit cells via exosomes. In the extracellular space, α-Syn-containing vesicles may be taken up via clathrin-dependent endocytosis and clathrin-independent, dynamin-dependent processes. α-Syn-containing exosomes are also thought to be taken up by cells. Some studies also suggest a role for TNTs in synuclein propagon spread (B). The transcellular transport of huntingtin (Htt) is not well understood. Htt aggregates may spontaneously diffuse across plasma membranes resulting in their transport. A receptor facilitating Htt uptake may exist, while TNTs may also play a role, as discussed in the main text (C). Amyloid precursor protein (APP) undergoes cleavage, releasing the Aβ peptide into the extracellular space. The uptake of aggregated Aβ is thought to occur in caveolae and lipid rafts in a dynamin-dependent manner. The involvement of receptor-mediated uptake, of exosomes, and possibly of TNT has also been proposed (D).

Tau is also secreted from cultured neurons through exosomes, but only if overexpressed (79, 80). α-Syn oligomers can be exported from primary neurons and neuronal cell lines into the extracellular milieu and are found within exosomes and on their surface. The α-Syn oligomeric fraction not associated with the exosomes seems to exert less toxicity on neighboring cells than those associated with the exosomal fraction (81).

While evidence suggests a role for exosomes in the cell-to-cell spread of protein ag-

gregates, proof of this concept is still lacking. The molecular mechanisms controlling exosome generation and release are largely unknown. Consequently, we lack the technical ability to control exosomal flow - a crucial limitation that essentially restricts research to observational studies. As a result, it is unclear what regulates the incorporation of oligomers into exosomes and whether the latter are epiphenomena or are causally involved in the transmission of aggregated pathogenic proteins.

Nonconventional Export

Nonconventional export is a broad term that describes the secretion of proteins by means other than the classical secretory pathways. PrP^C reaches the cell membrane via canonical cotranslational extrusion and maturation in vesicular compartments and is secreted through various endoproteolytic events (sometimes termed α and β cleavage, as well as C-terminal cleavage) or through hydrolysis of its glycolipid anchor, resulting in the release of soluble fragments of various sizes (82, 83). α and β cleavage occur around the hinge between the N-terminal FT and the globular domain of PrP^C and are extremely resilient to mutagenesis of the cleavage site (84), suggesting that cleavage may be performed by multiple sheddases. ADAM10 is thought to cleave PrP^C at residues 228/229 and ablation of the ADAM10 protease results in a dramatic decrease in the spread of prions from the site of injection (85). There have been suggestions that the shedding of the N terminus of PrP^C may have a neuroprotective role (86). Moreover, PrP^C-deficient mice suffer a demyelinating disease and transgenic expression of PrP^Cvariants that lack N-terminal cleavage fail to rescue the demyelination (87). This suggests that the FT of PrP^C, once cleaved, may travel to Schwann cells and activate a promyelinating pathway, perhaps by interacting with a hitherto unknown receptor.

Healthy cells secrete only small amounts of α-Syn, but secretion increases under stress (88). The secretion of tau may follow an unconventional route of export. Blocking endoplasmic reticulum (ER)-to-Golgi transport using Brefeldin A did not prevent the secretion of endogenous and overexpressed tau into the extracellular milieu, supporting the existence of an unconventional route whose details remain to be characterized.

The association of oligomers with membranes has raised the question of whether autophagy is involved in their transport. Autophagy plays a crucial role in defense against intracellular protein misfolding (89) and may play a key role in nonconventional protein export. Some unconventionally secreted proteins, including IL-1β, IL-18, HMGB1 in mammals, and α-PS1 (an α-integrin subunit), use autophagy for their transport into the extracellular space (90, 91). The Atg proteins and Golgi reassembly and stacking protein (GRASP) are important components of this system (92). The hijacking of autophagic organelles and intermediates could also explain how certain components of the cytosol can follow oligomers and larger aggregates into the extracellular space.

Exchange via Nanotubes

The exchange of oligomers and larger aggregates between cells may also occur via tunneling nanotubes (TNTs), which are present in many cell types including

neurons. TNTs are membranous channels based on F actin and microtubules with a diameter of 20-500 nm (93). TNTs can produce transient connections between cells; such connections are formed rapidly and are very fragile, and can serve as direct conduits for intracellular cargoes between cells (Figure 2).
Various cargoes are shipped through TNTs, including proteins, endosomes, and bulky organelles such as mitochondria, lysosomes, and Golgi vesicles (94). Early studies have shown that viruses including HIV (95) can hijack the TNT network to spread from one cell to another. PrP^{Sc} also appears to enter TNTs and move to target cells in a coculture system (96). Aβ was also shown to utilize TNTs for transfer between cells in coculture experiments and elicited toxicity in acceptor cells (97). Finally, coculture studies performed on murine tumor CAD5 cells showed that fluorescently labeled huntingtin aggregates access TNTs and transfer between cells (98).
Despite these interesting findings, our understanding of the potential role of TNTs in neurodegeneration (and in normal physiology) is limited. Some of the most elementary and pressing questions are as follows. Do all cytosolic protein aggregates use TNTs as a common mode of transport between cells? Are TNTs preferred over other routes? Are prion propagons (which are not necessarily identical with PrP^{Sc}) transferred by TNTs? Most importantly, are TNTs seen only in coculture systems or are they relevant to the pathogenesis of neurodegenerative diseases *in vivo*? Technological advances in the manipulation of TNTs may help in answering these fundamental questions.

Internalization from Plasma Membrane
On encountering a new cell, any propagon faces the challenging task of infecting it. In the case of intracellular aggregates, the difficulty is compounded by the challenge of crossing one or several lipid bilayers. Entry of bulky cargo into cells is classically achieved by receptor-mediated endocytosis (99). PrP^{C} is known to undergo clathrin-mediated endocytosis and is further sorted into late endosomes/MVBs or into recycling endosomes, which reroute it again to the plasma membrane (Figure 2A). Because of the technical difficulty of discriminating PrP^{C}, PrP^{Sc}, and propagons *in situ*, the compartments in which prion amplification occurs are not entirely clear, but many studies imply that it may occur throughout the endocytic pathway (75, 100, 101). Interestingly, prions can infect cells inhibited in classical endocytic pathways and micropinocytosis (102), suggesting the existence of other nonconventional endocytic pathways.
Within the brain, Aβ is primarily released into the extracellular compartment after its proteolytic release from APP. While the endocytic pathways that process Aβ aggregates are ill defined, there is evidence indicating that uptake is independent of clathrin but dependent on dynamin, the protein Rho, and cholesterol within the plasma membrane(103, 104). Cell-surface receptors including LRP1, NMDAR, and p75NTR appear to facilitate the entry of $A\beta_{42}$ into cells (105-107), where it has been shown to accumulate in late endosomes and lysosomes (108). Also, $A\beta_{40}$ and $A\beta_{42}$ tend to intrinsically interact with the plasma membrane owing to their charges. It is unclear whether they enter cells by membrane penetration,

analogously to the *Tat* peptide of HIV and antennapedia-domain proteins (109), or though different mechanisms (Figure 2D).
Tau aggregates can be internalized in a clathrin-independent, dynamin-dependent pathway depending on their aggregation state 104 and 110. Oligomers, but neither monomers nor large fibrils, are taken up by cultured primary neurons (111). Several studies have documented that α-Syn oligomers and fibrils can enter cells via both clathrin- and dynamin-dependent processes (112, 113). Expression of a dominant-negative form of dynamin (K44A Dynamin1) led to decreased uptake of α-Syn fibrils and monomers in cultured cells (113-115).
The mechanisms by which huntingtin oligomers and fibrils might move between cells have been less thoroughly studied. The binding of huntingtin fibrils to cell membranes is sensitive to proteolysis by trypsin, suggesting the presence of an as-yet-unidentified protein receptor (55). Colocalization studies have not revealed accumulation in any of the endocytic compartments, suggesting that they might enter cells directly across the plasma membrane, perhaps using hitherto unknown channels (Figure 2B).
α-Syn and huntingtin do not physiologically live inside vesicular compartments. Therefore, having gained access to the endosomal compartments, their oligomers still face the question of how to reach the cytosol where they can seed new reactions. α-Syn adopts an amphipathic α-helical structure that adapts to the curvature of cell-membrane lipids, similarly to endophilins (116). Analogous to the entry route of adenoviruses, α-Syn is thought to enter the cytosol through the rupture of endocytic vesicles, and its N-terminal domain has been shown to be crucial for translocation into the cytosol. Possibly, some oligomeric species of membrane-affine proteins may induce thinning of the lipid bilayer or even form membrane pores (117, 118), thereby facilitating entry into the cytoplasm (Figure 2C). Recent studies have also shown that release and uptake of α-Syn and huntingtin aggregates across neurons involves a transsynaptic mechanism (119-121), the details of which are not yet understood.

Concluding Remarks

The average life expectancy of the world population is increasing and age represents the strongest risk factor for protein aggregation diseases. Consequently, neurodegenerative diseases are expected to reach epidemic proportions in the coming decades. In this review we have focused on how misfolding of proteins leads to the generation of toxic oligomeric conformers, which are now largely considered the main culprits in many neurodegenerative disorders. While many important phenomena remain unexplained (see Outstanding Questions) and efficacious therapies for most PMDs are still lacking, there is reason for hope. Emerging technologies may facilitate the study of their cellular and molecular underpinnings, notably super-resolution microscopy (122), which allows quantitative monitoring of events occurring at the subcellular level *in vivo*. These techniques will provide details about aggregation and structural transitions in real time. CRISPR-based genome editing (123) is revolutionizing reverse genetics and is already being used

to validate genes involved in protein aggregate neurotoxicity. On the therapeutic front, most efforts are currently focused on using antibodies to target the spread of aggregates (124, 125) and after many setbacks some promising data are starting to appear. We have little doubt that we will see important theoretical advances in the near future, from which effective therapeutic approaches will eventually spawn.
How are the oligomeric seeds (the propagons) generated? Is there a molecular chaperone system in mammalian cells similar to that in yeast to break down the fibrils and generate more seeds? How does the frangibility of fibers influence the functioning of such chaperones?
Understanding how to prevent the generation of new seeds is key to suppressing oligomer propagation. Which pharmacophores are best suited to stabilizing aggregates and preventing their breakage?
How can aggregates alter various cell signaling cascades and eventually induce toxicity?

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Infiammazione e malattie neurodegenerative

15

Infiammazione e amiloidosi sistemica

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L'infiammazione svolge un ruolo diretto nella patogenesi di alcune forme di amiloidosi sistemica, quale l'amiloidosi reattiva (AA), e contribuisce alla progressione e al danno d'organo di altre, come l'amiloidosi da transtiretina (ATTR) e da beta-2 microglobulina (Ab2M), correlata alla dialisi. Di seguito sono descritti la patogenesi e gli aspetti clinici e terapeutici della amiloidosi AA.
L'amiloidosi sistemica AA è una complicanza a lungo termine di diverse malattie infiammatorie croniche, tra cui l'artrite reumatoide, la spondilite anchilosante, le sindromi autoinfiammatorie, la malattia di Crohn, i tumori maligni, e le condizioni predisponenti a infezioni ricorrenti, come la fibrosi cistica (1, 2) (Tabella 1). In circa un quinto dei pazienti che sviluppano amiloidosi AA non si riesce a determinare la malattia infiammatoria sottostante. La amiloidosi AA è stata per secoli la forma più frequente di amiloidosi sistemica, ma la sua incidenza, almeno nei paesi occidentali, è in costante riduzione negli ultimi anni, a causa di un migliore controllo delle condizioni infiammatorie sottostanti. Il danno d'organo deriva dal processo di deposizione extracellulare di frammenti proteolitici della proteina di fase acuta siero amiloide A (SAA) come fibrille di amiloide. Un'alta e persistente concentrazione della SAA nel siero è il prerequisito per lo sviluppo della amiloidosi AA. Tuttavia, solo una minoranza di pazienti con infiammazione di lunga data in realtà si presenta con questa complicanza, indicando l'esistenza di fattori modificanti la malattia.

Meccanismi molecolari della amiloidosi AA

La SAA è una proteina di fase acuta secreta dal fegato sotto il controllo trascrizionale di interleuchina-1 (IL-1), IL-6 e TNFa. La sua concentrazione aumenta fino a 1000 volte dopo una stimolazione infiammatoria. Se tali stimoli persistono, come avviene in molte malattie croniche, la concentrazione dell'SAA può raggiungere una soglia critica alla quale diventa incline all'aggregazione. Questa proprietà è comune ad altre proteine solubili che possono andare incontro a *misfolding*, aggregazione e, infine, generazione di fibrille di amiloide con conformazione a foglietto *cross*-β (3). In generale, almeno 32 diverse proteine sono note per essere in grado di formare fibrille amiloidi e depositarsi in forma sistemica o localizzata (4).

Tab. 1 - Condizioni infiammatorie associate alla amiloidosi AA.

Artriti infiammatorie Artrite reumatoide Spondilite anchilosante Malattia di Still dell'adulto Artrite idiopatica giovanile Artrite psoriasica Gotta **Malattie infiammatorie intestinali** Morbo di Crohn Colite ulcerosa **Immunodeficienze ereditarie ed acquisite** Immunodeficienza comune variabile Ipogammaglobulinemia Agammaglobulinemia *X-linked* Neutropenia ciclica HIV/AIDS **Altre** Obesità (?) Sarcoidosi SAPHO sindrome Sindrome di Schniztler	**Malattie neoplastiche** Malattia di Castleman Linfoma di Hodgkin Macroglobulinemia di Waldenström Leucemia a cellule capellute Adenoma epatico Carcinoma renale Adenocarcinoma del polmone Adenocarcinoma dell'intestino Mesotelioma **Infezioni croniche** Bronchiectasie Osteomielite Tubercolosi Pielonefrite cronica Lebbra Malattia di Whipple Ulcere cutanee croniche (decubito) Epatite B (?)	**Malattie autoinfiammatorie ereditarie** Febbre mediterranea familiare TRAPS Sindrome di Muckle-Wells Sindrome NOMID / CINCA Sindrome da iper-IgD **Vasculiti sistemiche** Malattia di Behcet Poliarterite nodosa Arterite a cellule giganti Arterite di Takayasu Polimialgia reumatica **Condizioni predisponenti alle infezioni croniche** Fibrosi cistica Epidermolisi bollosa Abuso di droghe endovena By-pass digiuno-ileale Paraplegia

Due diverse isoforme di SAA, SAA1 e SAA2, sono alla base dell'aumento dei livelli di SAA durante la risposta di fase acuta. Nell'uomo sono noti tre principali alleli *SAA1*, indicati come *SAA1.1, SAA1.3 e SAA1.5*, che si differenziano per le sostituzioni di amminoacidi singoli ai codoni 52 e 57. In soggetti giapponesi, la presenza di omozigosi per *SAA1.3* aumenta significativamente il rischio di amiloidosi ed è anche associata ad un più breve periodo di latenza prima dell'esordio, a manifestazioni cliniche più severe e ridotta sopravvivenza (5). I soggetti caucasici omozigoti per *SAA1.1* sviluppano amiloidosi AA da tre a sette volte più frequentemente di altri genotipi (25). Molte domande rimangono aperte sulle ragioni biologiche di questa discrepanza tra le diverse popolazioni e sui meccanismi molecolari con cui questi alleli influenzano in modo variabile lo sviluppo di amiloidosi AA (6).

Il primo passo del processo di fibrillazione è il parziale svolgimento del precursore amiloidogenico in una forma solubile, monomerica, con conformazione nonnativa che si auto-assembla in aggregati oligomerici caratterizzati da una molto rapida cinetica di associazione/dissociazione, e altamente sensibili all'interazione con componenti della matrice extracellulare, alla proteolisi limitata, al pH, e ad altri fattori del microambiente tissutale (Figura 1). Alcuni di questi oligomeri pre-amiloide possono esercitare un effetto citotossico diretto. Nella amiloidosi AA, le fibrille sono invariabilmente formate da frammenti N-terminali che comprendono i primi 66-76 aminoacidi di SAA, indicando un ruolo fondamentale del rimodellamento proteolitico. Le interazioni con i glicosaminoglicani (soprattutto con l'eparansolfato) promuovono il *misfolding* e l'aggregazione di SAA sia in

vitro che in vivo e sono in grado di accelerare il processo amiloidogenico agendo come impalcatura per la polimerizzazione (7). Una pentraxina, la SAP, è ubiquitariamente presente in tutti i depositi di amiloide ed è per questo sfruttata sia per l'*imaging* sia come bersaglio terapeutico. Il processo di fibrillazione è un processo sfavorevole in cui proteine devono superare una barriera termodinamica e cinetica e quindi è atteso che si verifichi in tempi lunghi (*lag phase*). Tuttavia, una volta formato un primo nucleo, questo agisce come un seme (*seed*) favorendo l'ulteriore polimerizzazione e la conseguente crescita accelerata dei depositi di amiloide. Questo modello di nucleazione avviene per la maggior parte delle proteine amiloidogeniche ed è alla base del rapido peggioramento che spesso si osserva in questi pazienti a seguito di processi infettivi/infiammatori intercorrenti. La rilevanza patogenetica del meccanismo di *seeding* come un meccanismo generale sottostante le malattie amiloidi è supportato anche da crescenti evidenze in modelli animali (8). Si è ipotizzato che i meccanismi di *seeding* siano coinvolti non solo nella trasmissione e accelerazione della amiloidogenesi, ma potrebbero anche contribuire alla diffusione della malattia a organi bersaglio, giocando un ruolo importante nel determinare la specificità tissutale (9).

Il rene, il fegato, la milza, la tiroide e il sistema nervoso autonomo sono i principali siti di deposizione di amiloide. Il cuore è coinvolto raramente e sempre nelle fasi avanzate della malattia. Il decorso clinico è dominato dal coinvolgimento renale, con proteinuria importante e progressiva perdita della funzione renale, che porta alla dialisi nel 40% dei casi (1). La funzione renale, il carico di amiloide e la mortalità sono significativamente correlati con i livelli di SAA durante il decorso della malattia (10).

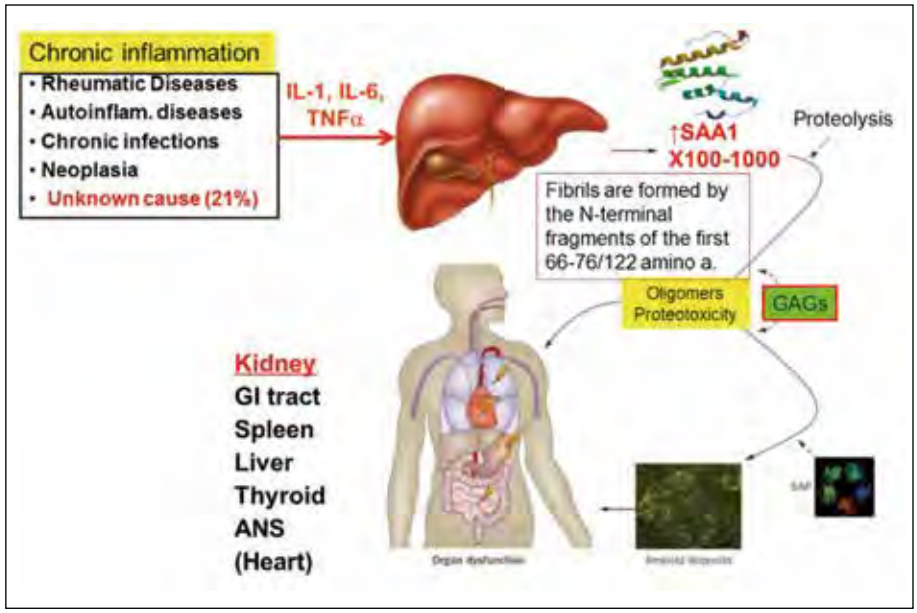

Fig. 1 - Cascata patogenetica della amiloidosi AA.

Terapie attuali ed emergenti

Il fondamento della terapia è il controllo della condizione infiammatoria sottostante. Questo è ora grandemente facilitato dalla introduzione delle cosiddette terapie biologiche anti-citochine. Oltre ai corticosteroidi e ai farmaci citostatici, il trattamento con anticorpi monoclonali contro citochine, particolarmente TNF e IL-6, è efficace in molti casi nel controllare la malattia di base e nel ridurre marcatamente la concentrazione della SAA (1, 2). La normalizzazione dei livelli di SAA è associata a progressiva, anche se lenta, regressione dei depositi di amiloide, miglioramento della proteinuria e della funzione renale e ad allungamento della sopravvivenza (10). Misurazioni seriali dei livelli di SAA, insieme con il monitoraggio dei biomarcatori renali (albumina urinaria delle 24 ore e clearance della creatinina), rappresentano la pietra angolare per il monitoraggio della risposta alla terapia. I pazienti con amiloidosi AA e insufficienza renale all'ultimo stadio, ma con la malattia infiammatoria sottostante completamente sotto controllo, possono essere candidati al trapianto renale. Sono stati riportati bassi tassi di recidiva amiloide nel trapianto (4-14%), ma questi pazienti richiedono un'attenta gestione delle complicanze cardiovascolari e infettive (11).

L' eprodisato è un composto con basso peso molecolare, strutturalmente simile all'eparan-solfato. Si lega in modo competitivo ai siti di legame dell'SAA sui glicosaminoglicani, inibendo così la polimerizzazione in fibrille. Questo composto ha ridotto significativamente la deposizione di amiloide in un modello murino di AA (12). In un trial clinico di fase II/III, condotto su 180 pazienti, il trattamento di 2 anni con eprodisato ha portato ad una riduzione significativa della velocità di declino della creatinina rispetto al placebo, ma nessuna significativa riduzione della proteinuria, o effetto sulla sopravvivenza (13). Un secondo studio di conferma di fase III è in corso (NCT01215747).

Recentemente è stato sviluppato un trattamento combinato con un farmaco in grado di eliminare la SAP circolante (CPHPC) con un anticorpo monoclonale diretto contro la SAP. Questa combinazione si è dimostrata efficace nell'accelerare il riassorbimento dei depositi viscerali di amiloide nel modello murino (14) e nei pazienti in uno studio clinico di phase I/II (15).

Il knockdown della SAA tramite oligonucleotidi anti-senso è stato sperimentato con successo nei topi, con riduzione della gravità della amiloidosi AA sperimentale (16). Questo approccio non è stato ancora tradotto in clinica, ma potrebbe essere particolarmente utile per quei casi di amiloidosi AA nei quali la condizione infiammatoria di base non può essere identificata e trattata con successo.

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Infiammazione e neurodegenerazione nella sclerosi multipla

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La sclerosi multipla (SM) è una patologia cronica del sistema nervoso centrale (SNC) caratterizzata da infiammazione, demielinizzazione e neurodegenerazione (Compston and Coles, 2008; Yadav et al., 2015). La malattia colpisce i giovani adulti normalmente prima dei 40 anni di età, in maggioranza donne, e conduce a disabilità invalidante con un forte impatto negativo sulla qualità della vita (Compston and Coles, 2008; Yadav et al., 2015). Nella maggior parte dei pazienti la SM inizia con un decorso recidivante-remittente (RR) caratterizzato da episodi acuti di sintomi neurologici seguiti dal recupero spontaneo delle funzioni (Compston and Coles, 2008). Dopo mediamente 15-20 anni di malattia la maggior parte dei pazienti sviluppa la forma progressiva di SM, costituita da un graduale deterioramento delle funzioni neurologiche dovuto a neurodegenerazione senza episodi infiammatori acuti riconoscibili (Compston and Coles, 2008). Il 10-15% dei pazienti con SM ha un decorso progressivo già dall'esordio della malattia (Compston and Coles, 2008). Una serie di studi clinici ha mostrato come il numero di ricadute acute durante la fase RR non determini né il rischio di entrare nella fase progressiva né la severità di progressione (Scalfari et al., 2010; Scalfari et al., 2013), suggerendo l'ipotesi che la SM sia una malattia causata inizialmente dall'infiammazione e più avanti dalla neurodegenerazione. Tuttavia evidenze recenti di neuroradiologia e neuropatologia indicano come la neurodegenerazione sia chiaramente presente già nelle fasi iniziali di malattia e che la transizione dalla forma RR a quella progressiva abbia luogo quando la compensazione del danno assonale eccede la sua capacità. Infatti, l'atrofia cerebrale e spinale, uno dei più importanti marcatori radiologici per la neurodegenerazione, può essere rilevata nei pazienti con sindromi cliniche isolate suggestive di SM ed è a carico sia della sostanza bianca che della sostanza grigia (Chard et al., 2002; Barkhof et al., 2009). L'analisi istologica delle lesioni infiammatorie ha confermato la presenza di cellule immunitarie nel parenchima cerebrale e l'attivazione delle cellule gliali, e messo in luce il danno assonale e la ridotta densità neuronale nella SM (Bjartmar et al., 2000; Kornek et al., 2000; Bitsch et al., 2000; De Luca et al., 2004; Kutzelnigg et al., 2005; Frischer et al., 2009; Lassmann, 2010; Lassmann, 2013; Lassmann, 2014). Mentre le lesioni a carico della sostanza bianca sono rilevabili in tutte le forme di SM, quelle nella sostanza grigia corticale sono più

frequenti nelle forme progressive (Peterson et al., 2001; Vercellino et al., 2005; Lucchinetti et al., 2011), suggerendo un loro ruolo nel determinare disabilità irreversibile. A supporto di questa ipotesi, la massiccia perdita neuronale nelle lesioni della sostanza grigia corticale è associata con la diffusa infiammazione meningeale, che a sua volta correla con la progressione della disabilità (Howell et al., 2011; Choi et al., 2012). Queste osservazioni suggeriscono quindi che i fattori solubili prodotti dalle cellule infiammatorie nelle meningi abbiano un ruolo chiave nella neurodegenerazione corticale.

La conoscenza dei processi patogenetici della SM ci deriva dallo studio del suo modello animale, l'encefalite autoimmune sperimentale (EAS), che riproduce alcuni aspetti clinici e neuropatologici della SM (Steinman and Zamvil, 2005; Kipp et al., 2012). Molteplici sono i processi implicati nella neurodegenerazione durante l'EAS ed includono la produzione di specie reattive dell'ossigeno e dell'azoto, di ipossia, di citochine e glutammato (Pitt et al., 2000; Smith et al., 2001; Aboul-Enein et al., 2003; Vercellino et al., 2007; Mahad et al., 2008; Trapp and Stys, 2009; Haider et al., 2011; Fischer et al., 2012; Colombo et al., 2012; Colombo et al., 2014). Questi, in combinazione con la mancanza di supporto neuroprotettivo, determinano stress ossidativo, demielinizzazione, danno mitocondriale e deficit energetico che a loro volta causano la morte neuronale. Le lesioni di SM presentano frammentazione del DNA tipica del processo apoptotico nei neuroni nelle aree di attiva demielinizzazione, nelle lesioni corticali inattive ed anche nella sostanza bianca apparentemente normale (Peterson et al., 2001; Fischer et al., 2013). Inoltre hanno evidenza di degenerazione Walleriana, un processo di frammentazione assonale innescato dalla sezione in tutto il suo spessore dell'assone (Dziedzic et al., 2010).

Questi processi neurodegenerativi non sono autonomi nella cellula neuronale, ma al contrario sono fortemente determinati dall'interazione del neurone con gli altri tipi cellulari residenti nel SNC e quelli infiltranti nelle fasi infiammatorie. A questo proposito, il nostro laboratorio ha messo in luce il ruolo cruciale dell'astrocita nella neurodegenerazione durante la neuroinfiammazione (Colombo and Farina, 2012; Colombo et al., 2012; Colombo et al., 2014). È noto che la generazione e la trasmissione dell'informazione sotto forma di impulso nervoso nel SNC siano a carico della rete neuronale. Tuttavia gli astrociti, che costituiscono la popolazione gliale più numerosa, regolano il funzionamento della rete neuronale in quanto forniscono supporto trofico e metabolico ai neuroni e determinano la formazione e funzione delle sinapsi neuronali. Gli astrociti hanno inoltre un repertorio di strumenti di riconoscimento di segnali di pericolo, che fa sì che in caso di danno tissutale possano prontamente rispondere ed attivare strategie di contenimento della lesione mediante la formazione di tessuto cicatriziale, all'interno del quale ha luogo una reazione infiammatoria acuta necessaria per la risoluzione della lesione e la ricostruzione del tessuto (Farina et al., 2007; Sofroniew, 2009; Cordiglieri and Farina, 2010; Sofroniew and Vinters, 2010). È quindi evidente che la disregolazione dell'attività dell'astrocita può essere pericolosa per il funzionamento del SNC e che la conoscenza delle modalità con cui gli astrociti contribuiscono ai processi patogenetici è fondamentale per

lo sviluppo di approcci terapeutici efficaci. Per esempio, nel 2012 abbiamo descritto la forte espressione del recettore TrkB delle neurotrofine sugli astrociti nelle lesioni da sclerosi multipla e nel suo modello animale (Colombo and Farina, 2012; Colombo et al., 2012). Le neurotrofine costituiscono una famiglia di fattori di crescita (Reichardt, 2006), il cui capostitipe è il Nerve Growth Factor su cui tanto ha lavorato il premio Nobel Rita Levi-Montalcini. Queste molecole, essendo importanti per la sopravvivenza, lo sviluppo e la funzione dei neuroni, sono ritenute benefiche per la neuroprotezione e la neurorigenerazione (Reichardt, 2006). Tuttavia, studiando il modello animale della sclerosi multipla in topi geneticamente modificati, abbiamo riscontrato un nuovo e sorprendente meccanismo: la forte espressione del recettore TrkB sugli astrociti, e quindi la maggiore sensibilità alle neurotrofine, risulta nella produzione di ossido nitrico, un responsabile cruciale dei danni da sclerosi multipla poiché porta alla morte dei neuroni (Colombo and Farina, 2012; Colombo et al., 2012). Infatti, le analisi in vitro e in vivo hanno dimostrato come questo processo patologico crei un ambiente permissivo all'infiltrazione di cellule immunitarie e alla neurodegenerazione (Colombo and Farina, 2012; Colombo et al., 2012). Risulta perciò verosimile che una forte espressione del recettore TrkB sull'astrocita contribuisca in maniera decisiva ai danni neuronali propri della sclerosi multipla, dato il suo ruolo nella produzione di ossido nitrico. Diventa quindi importante usare terapie che limitino la produzione di ossido nitrico da parte degli astrociti al fine di bloccare la neurodegenerazione. A questo proposito abbiamo più recentemente individuato una via di trasduzione del segnale nell'astrocita che porta alla sintesi di ossido nitrico e che viene bloccata da un farmaco attualmente in uso nella SM (Colombo et al., 2014). Fingolimod è il primo farmaco a somministrazione orale approvato per il trattamento della sclerosi multipla (Brinkmann et al., 2010). Agisce legando i recettori per la Sfingosina-1-fosfato (S1P), un lipide mediatore della migrazione dei linfociti dagli organi linfoidi verso il torrente circolatorio (Spiegel and Milstien, 2011). Le persone con SM traggono beneficio dal trattamento del Fingolimod grazie agli effetti bloccanti del farmaco sulla motilità delle cellule immunitarie (Brinkmann et al., 2010). L'osservazione iniziale del nostro studio è stata l'aumentata espressione dei recettori per le citochine infiammatorie IL1 e IL17 e per S1P sugli astrociti nelle placche di sclerosi multipla (Colombo et al., 2014). La stessa area presentava tracce dell'azione dell'ossido nitrico (Colombo et al., 2014). Indagini condotte in vitro e su modelli animali hanno dimostrato come gli astrociti rispondano alla stimolazione con IL1 e IL17 rilasciando ossido nitrico che induce neurodegenerazione (Colombo et al., 2014). Per fare ciò però hanno bisogno dell'attivazione della via di trasduzione del segnale di S1P. È stato importante rilevare come fingolimod, bloccando i recettori di S1P, inibisse anche l'attivazione evocata dalle citochine infiammatorie e il conseguente rilascio di ossido nitrico sia in vitro che in vivo (Colombo et al., 2014). Questa importante scoperta avvalora l'ipotesi di un effetto neuroprotettivo del fingolimod nel sistema nervoso centrale che si esplica però non direttamente sul neurone ma tramite la modulazione dell'attività dell'astrocita. Quindi la sclerosi multipla non è semplicemente il risultato di una reazione immunitaria

nel SNC con impatto sull'oligodendrocita inizialmente e sul neurone poi, bensì il risultato dell'interazione complessa tra tutti i protagonisti cellulari presenti nel SNC infiammato. È evidente come le terapie ad azione anti-infiammatoria, così efficaci nella forma RR di malattia, non siano adeguate per bloccare la neurodegenerazione e favorire la neurorigenerazione e rimielinizzazione nelle forme progressive di SM. La sfida dei prossimi 10 anni sarà di sviluppare strategie nuove che tengano in considerazione tutte le componenti cellulari coinvolte nella patogenesi della SM e che siano in grado di riprogrammare il microambiente ai fini del riparo del tessuto cerebrale.

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The role of inflammation in Alzheimer's disease

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Alzheimer's disease - an enormous challenge for the society

Alzheimer's disease (AD) is the most common form of dementia and it affects more than 35 million people worldwide (1). Based on the aging population, it is estimated that by 2030 nearly 66 million people will have AD (thus in the next 17 years the number of people will double) and by 2050 Alzheimer's will be a global epidemic with rates exceeding 115 million individuals. AD drives to death within 3 to 9 years after diagnosis and THERE IS NO CURE FOR THE DISEASE. Current drugs provide symptomatic benefit for up to 1 year for some patients, but there are no disease-modifying therapies.

AD is characterized by a progressive deterioration of cognitive function, and the neuropathological features include amyloid β (Aβ) neuritic plaques, neurofibrillary tangles (NFTs) comprising aggregates of hyperphosphorylated microtubule tau protein, amyloid angiopathy, and the loss of neurons and synapses[1].

The amyloid cascade hypothesis has been the major pathogenic concept in the field of AD research for the past decades. It states that the pathological sequence of events leading to AD is characterized by the accumulation of Aβ peptides resulting from the aberrant processing of amyloid precursor protein (APP) APP and dysfunctional Aβ clearance, followed by the deposition of NFTs and the onset of synaptic and neuronal dysfunction and loss. Aβ peptides originate from proteolysis of the APP by the sequential enzymatic actions of beta-site amyloid precursor protein-cleaving enzyme 1 (BACE-1), a β-secretase, and γ-secretase, a protein complex with presenilin 1 (PS1) at its catalytic core. An imbalance between production and clearance, and aggregation of peptides, causes Aβ to accumulate, and this excess may be the initiating factor in Alzheimer's disease. A central mechanism underlying the formation of both amyloid plaques and NFTs in AD is therefore pathogenic cerebral protein aggregation. Though both amyloid plaques and aggregated tau are part of the neuropathological definition of the disease, numerous studies suggest that soluble oligomeric forms of Aβ and tau are the chief mediators of cytotoxicity in AD (2).

Intense research efforts in the past decades to understand the pathogenesis of neurodegenerative diseases and design effective therapeutics have been focused mainly on neurons and other CNS resident cells. Although this "neurocentric" view

has contributed to our understanding of neuronal dysfunction, death pathways, and accumulation of proteinaceous aggregates during chronic neurodegenerative processes, this approach has not resulted in disease-modifying therapeutics. This suggests that the pathogenesis of neurodegenerative disorders is more complex than previously thought, and that the lack of success of neurocentric-based therapies may be due to the participation of non-neuronal cells in the disease process.

Inflammation in Alzheimer's disease

AD pathology is also characterized by an inflammatory response, which is considered to be primarily driven by cytokines and the brain's intrinsic myeloid cells known as microglia (3). It is now widely accepted that microglia-mediated neuroinflammatory responses may promote neurodegeneration in AD (1, 3). Microglial activation precedes neuropil loss in patients with AD and recent genome-wide association studies have revealed that microglial genes such as *CD33*, *TREM2* and *HLA-DR* are associated with susceptibility to late-onset disease (3). Moreover, in response to Aβ or neurofibrillary tangles, microglial cells produce pro-inflammatory cytokines, chemokines and complement peptides, which potentially recruit leukocyte subpopulations to the brain. Aβ also stimulates microglia to produce reactive nitrogen intermediates such as nitric oxide (NO) and reactive oxygen species (ROS) and the resulting oxidative stress induces neuronal damage (3). Overactive microglia can therefore induce significant and highly-detrimental neurotoxic effects, hence the attenuation of the microglial response has been proposed as a potential therapeutic approach in AD. The cognitive status of AD patients is inversely correlated with microglial activation, and recent studies have shown that microglia play a role in learning and learning-associated synaptic structural remodelling.

Chronic inflammatory disorders in humans, including atherosclerosis, obesity, diabetes and periodontitis, represent either risk factors for or correlate with the risk of late-onset AD (1). Furthermore, previous studies have suggested that inflammation may induce the cytoskeletal abnormalities associated with phosphorylated tau protein, thus disrupting axonal transport[4]. Accordingly, epidemiological studies have suggested that nonsteroidal anti-inflammatory drugs (NSAIDs) reduce the risk of AD, thus confirming its link with inflammation (1, 4). Even so, several prospective placebo-controlled trials to test the efficacy of NSAIDs against AD have failed, suggesting that specific inflammation pathways need to be identified and targeted.

Vascular inflammation and a dysfunctional blood-brain-barrier (BBB) have been implicated in the pathogenesis of AD (5). Aβ induces the expression of adhesion molecules on brain endothelial cells, potentially facilitating leukocyte adhesion and subsequent transmigration (5). Blood-derived leukocyte subpopulations, including lymphocytes, monocytes and neutrophils, have been identified in the brains of patients with AD and in some corresponding animal models (5, 6). Whereas blood monocytes have been associated with Aβ clearance, the role of other circulating leukocytes in the induction of neuropathological changes and memory deficit associated with AD is unclear.

Neutrophil trafficking in Alzheimer's disease

The migration of leukocytes from blood vessels into the central nervous system (CNS) is a key event in the pathogenesis of neurological diseases involving acute and chronic inflammation. Leukocyte extravasation is a finely regulated sequence of events controlled by adhesion molecules and activating factors (5). It is often described in terms of the following four "classical" steps:

1) capture (tethering) and rolling, which are mediated by low affinity interactions between selectins and mucins, and between integrins and members of the immunoglobulin (Ig) superfamily;
2) activation, during which signalling through the $G\alpha_i$ pathway is induced by chemoattractants and leads to the activation of integrins;
3) arrest, which is mediated by leukocyte integrins and their endothelial counterligands;
4) diapedesis/transmigration (5).

More recently, several additional steps have been defined, including slow rolling, adhesion strengthening and spreading, intravascular crawling, and finally transcellular and paracellular transmigration.

Over the last 20 years, leukocyte migration in the CNS has been investigated almost exclusively in the context of stroke and multiple sclerosis (MS). Experimental models of ischemic stroke have led to the characterization of adhesion molecules controlling leukocyte migration during acute inflammation, whereas experimental autoimmune encephalomyelitis (EAE), the animal model of MS, has provided similar data for chronic inflammation. Such experiments have led to clinical trials of anti-leukocyte adhesion therapy, with consistently positive outcomes in human subjects with MS, showing that interference with leukocyte adhesion can ameliorate chronic inflammatory CNS diseases.

Neutrophils are considered the main protagonists in the first line of defence during acute inflammatory conditions.

However, neutrophils have gained more attention recently in the context of chronic inflammation, e.g. atherosclerosis, adipose tissue inflammation, rheumatoid arthritis and multiple sclerosis (7). Neutrophils are now regarded as key players that directly affect the pathogenesis of chronic inflammatory diseases. Neutrophils are highly reactive cells releasing reactive oxygen species, enzymes, neutrophil extracellular traps (NETs) and cytokines, and can thus cause collateral tissue damage even in the absence of substantial accumulation within tissues during chronic inflammation. Previous studies as well as our own data have shown that neutrophil adhesion in CNS venules plays an important role in the pathogenesis of CNS disorders associated with sterile inflammation such as ischemic stroke and epilepsy (8, 9).

Using mice with five familial AD (5xFAD) mutations presenting amyloid pathology, and 3xTgAD mice with both amyloid and tau pathology, we found neutrophils extravasating in areas with amyloid Aβ deposits (Figure 1), and releasing NETs (10). Aβ142 peptide triggered the LFA-1 integrin high-affinity state and rapid neutrophil adhesion.

Two-photon microscopy experiments showed that LFA-1 integrin controls neutrophil extravasation and intraparenchymal motility (Fig. 2).

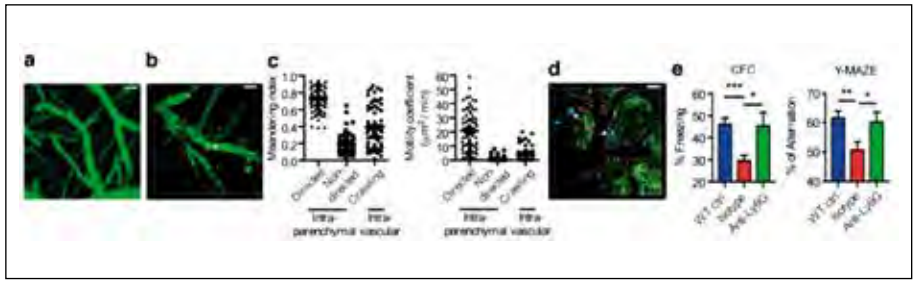

Fig. 1 - **Neutrophil migrate in the brain of mice with Alzheimer's-like disease and promote cognitive decline. a, b.** Neutrophils were labeled in red with CMTPX followed by intravenous injection. Blood vessels were labeled in green using 525-nm Qdots. Representative two-photon images of (a) WT control animals and (b) 5xFAD mice showing migrating cells inside the parenchyma, or adherent and crawling cells inside the blood vessels. Scale bar =50 μm. c. Movement parameters of intravascular and intraparenchymal neutrophils captured with Imaris software. d. Two-photon images showing neutrophils labeled in red with and migrating in the 5xFAD-YFPH mouse cortex (neurons are green). Aβ were labeled in blue following intravenous injection of MeO-X04. The vessel edge was artificially tracked. Scale bar =50 μm. e. Neutrophil depletion was carried out with an anti-Ly6G antibody (1A8) for 4 weeks starting at 6 months of age in 3xTg-AD mice. Healthy C57BL/6 mice were used as wild-type controls (WT ctrl). Left panel shows the results from the contextual fear-conditioning test (CFC); right panel shows the percentage of spontaneous alternation performance in the Y-maze test. Values represent median ± SEM from a representative experiment with 12-14 mice/condition from a series of three with similar results (*P<0.05 and **P<0.005). From Zenaro et al., Nat. Med. 2015.

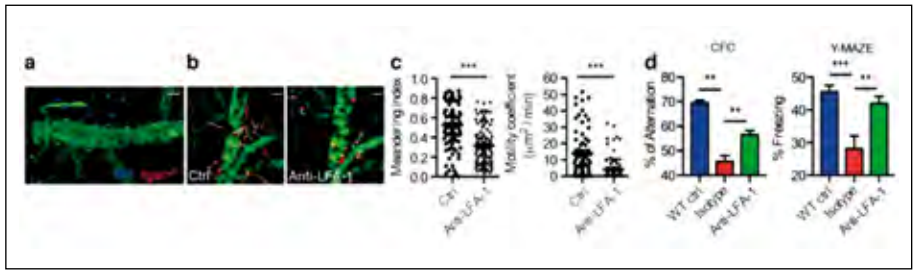

Fig. 2 - **LFA-1 integrin controls neutrophil trafficking and has a role in the induction of cognitive decline. a.** Blood vessels were labeled in green using 525-nm non-targeted Qdots. WT neutrophils (blue) invaded the brain parenchyma of 5xFAD mice, whereas Itgal$^{-/-}$ neutrophils deficient of LFA-1 integrin (red cells) were not able to extravasate. Scale bar =50 μm. b, c. Neutrophils were isolated from WT control mice, labeled and injected into the tail vein of 4-month-old 5xFAD mice 24 h before image acquisition. b. Blood cortical vessels were labeled in green using 525-nm non-targeted Qdots. After 1-2 h monitoring neutrophil movement (control untreated cells, Ctrl), LFA-1 was blocked by administering a single iv dose of anti-LFA-1 mAb (TIB213). After 20 min, image acquisition was restarted in the same areas to determine how the LFA-1 blockade affected neutrophil motility. Scale bar =30 μm. c. Analysis of cell motility parameters obtained with Imaris software showing a reduction of neutrophil movement after the injection of anti-LFA-1 antibody. (***P<0.0005). d. LFA-1 integrin was blocked by treating 3xTg-AD mice with the TIB213 antibody for 4 weeks starting at 6 months of age. An isotype-matched antibody was used as control. C57BL/6 littermates were used as wild-type controls (WT ctrl). The results from CFC and Y maze behavior test show learning and memory improvement after anti-LFA-1 treatment. Values represent the median ± SEM in each group. Data are from one representative experiment with 12–14 mice/condition (**P<0.005 and ***P<0.0005) (from Zenaro et al., Nat. Med. 2015).

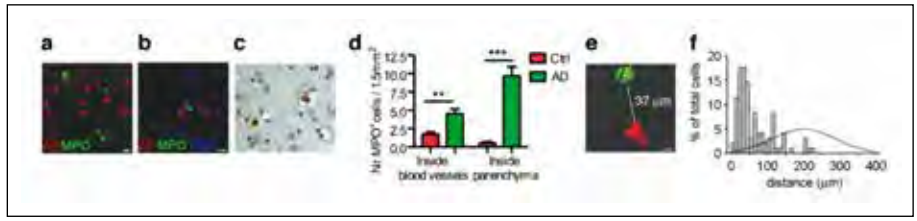

Fig. 3 - **Neutrophils adhere in brain vessels, migrate within the parenchyma in proximity of Aβ deposits in patients with AD. a, b.** Paraffin-embedded sections of human cortex and hippocampus from AD patients were labeled with antibodies specific for Aβ (red) and MPO (green), or the nuclear stain DAPI (blue). In (a), the presence of MPO^+ cells migrating inside the brain parenchyma is shown. b. MPO^+ cells were also found adhering to the blood vessel walls near areas with Aβ deposits. Scale bar =10 µm. c. Representative image of $CD66b^+$ cells counterstained with hematoxylin in the cerebral parenchyma of an AD patient. White arrows indicate intraparenchymally migrated neutrophils. Scale bar =20 µm. d. Quantification of MPO+ cells in healthy controls (n = 11) and AD patients (n =11). Values represent the number of cells per mm^2 of tissue ± SEM (**P<0.005; ***P<0.0005). (d) The distance between an MPO^+ cell and the adjacent plaque is shown by the white line, which connects the center of the amyloid plaque (red) and the center of the MPO^+ cell (green). (e) Distribution of distances between MPO^+ cells and adjacent plaques in AD patients (bar graphs) compared to the distribution of random distances between MPO^+ cells and plaques generated using Monte Carlo simulations (Gaussian curve in black) (P<0.00001; Anderson-Darling test). From Zenaro et al., Nat. Med. 2015.

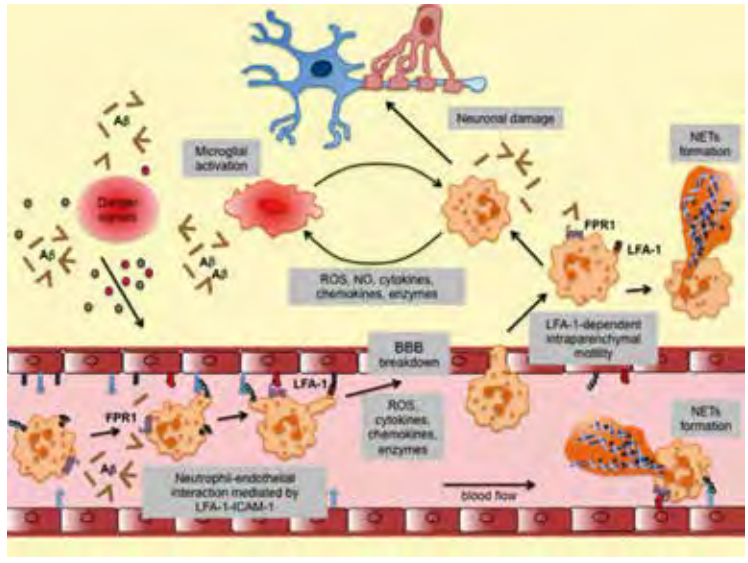

Fig. 4 - **The role of vascular inflammation and neutrophils in AD.** Aβ or danger signals released from brain cells lead to the activation of the endothelium, inducing the expression of adhesion molecules and chemoattractants. Vascular inflammation favors neutrophil endothelium adhesion interactions mediated by LFA-1, β2 integrin, ICAM-1 and potentially other adhesion molecules. Greater Aβ levels in the blood may prime circulating neutrophils through formyl peptide receptors (FPRs). Chemoattractants and Aβ on the endothelial surface may activate the LFA-1 high-affinity state and trigger rapid intravascular adhesion through FPRs and other G-protein-coupled receptors. Neutrophils that have adhered and spread inside the blood vessels may release reactive oxygen species (ROS), cytokines, chemokines and enzymes contributing to the destruction of the blood-brain barrier. Cells adhering within the vasculature release neutrophil extracellular traps (NETs), comprising decondensed chromatin and active proteases, including neutrophil elastase and myeloperoxidase. Extravasated neutrophil movement inside the parenchyma is controlled by LFA-1 and potentially by Aβ/FPRs and other chemoattractants/GPCRs and may harm neural cells by releasing NETs, enzymes, ROS, nitric oxide (NO), cytokines and chemokines. Intrapa-

renchymal Aβ may activate neutrophil integrins by binding to FPRs. The neutrophil–microglial interaction may create several feedback loops that amplify and sustain their activation, potentially representing a negative driving force in the pathogenesis of Alzheimer's disease. Neutrophils migrating within the parenchyma also contribute to the formation of Aβ deposits, tau phosphorylation and synaptic dysfunction, suggesting they play a pivotal role in the cognitive decline related to Alzheimer's disease (from Zenaro et al., Nat. Med. 2015).

Neutrophil depletion or the inhibition of neutrophil trafficking using genetic ablation or an anti-LFA-1 antibody dramatically reduced the neuropathological hallmarks of disease and rescued memory loss (Fig. 2). In AD patients, our results showed that neutrophils adhered and spread inside brain venules or migrated into the parenchyma in proximity of amyloid deposits (Fig. 3) and released NETs in larger numbers than in control subjects (10). Together, these results support the idea that circulating leukocytes have a pivotal in AD and that inhibition of immune cell trafficking may represent a new therapeutic strategy to address AD.

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Infiammazione e neurodegenerazione nella malattia di Parkinson

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Abstract

Parkinson's disease (PD) is a chronic neurodegenerative disease associated to both environmental and genetic etiological factors. It is increasingly reported in the literature that inflammation may be a pathogenic factor in the onset or more likely in the progression of familial and sporadic PD. This has triggered a large number of investigations aimed to define the role of inflammatory factors in PD. In vivo evidence for inflammation in PD spans from deregulated molecular mediators such as cytokines, complement system and their receptors, resident microglial activation, peripheral immune cells invasion, and modified composition and phenotype of peripheral immune cells. In this lecture we will explore how the current experimental approaches attempt to unravel the pathophysiologic role of immunity in PD with the potential to develop novel therapeutic targets for PD.

Parkinson's disease (PD) is a neurodegenerative disorder, which has as pathognomonic signs loss of dopaminergic neurons in the *substantia nigra* (SN) and the presence of alpha-synuclein deposits in diverse regions of the brain (1). The decrease of nigral neurons results in reduced striatal dopamine content, which leads to the characteristic motor symptoms: bradykinesia, rigidity and tremor. In addition, non-dopaminergic neurons seem to degenerate during the progression of the disease and account for dopamine resistant symptoms such as olfactory dysfunction, autonomic dysfunction, sleep disorders and sensory manifestations (2). The mechanism(s) which lead to neurodegeneration in PD are not fully unravel, and probably for this very reason, they have been associated to a multitude of processes such as: alpha-synuclein oligomerization (3), deficiency in mitochondrial activity (4), oxidative stress, increased metal ions content and proteins dis-homeostasis (5). Of relevance to the subject of this lecture, epidemiological, neurogenetics and post- mortem studies, suggest a contribution of neuroinflammatory processes in the progression of Parkinson disease. However, it is worth noting that while data provided by experimental models of the disease suggest an involvement of neuroinflammation, the latter is unlikely to be the primary causes of cell death in PD. A consideration that, as already reported in the literature (and probably discussed in other contribution to this meeting), may apply to other neurodegenerative disorders including Alzheimer's disease, Huntington's disease,

amyotrophic lateral sclerosis and progressive supranuclear palsy (6). In this lecture, we will explore the recent literature on the proposed role of neuroinflammation in Parkinson's disease and we will analyse the most recent reports on possible therapeutic approaches.

Soluble Signalling Proteins and Cytokines

As mentioned above, there are several indications that neuroinflammation may be a concurring factor of DA neuron degeneration. These include studies in which polyinosinic: polycytidylic acid (a Toll-like Receptor 3 (TLR) agonist) and Lipopolysaccharides (a Toll-like Receptor 4 agonist) were used to induce loss of DA neuron at the SN *pars compacta* in mouse (7, 8). Further support to the role of neuroinflammation emerges from experiments in which PD associated genes, such as PARK8 (the gene coding for Leucine-rich repeat kinase 2 (LRRK2) a multi-domain kinase) and SNCA (the gene coding for alpha-synuclein) were differentially expressed in immune cells. In this frame, a study from our laboratory provides indications for a role of LRRK2 in microglia in both activation and sustainment of neuroinflammation and in controlling of NF-κB p50 inhibitory signalling (9). A very recent and comprehensive review by La Voie et al., explores the relation among PD-associated mutations in LRRK2 and microglial biology in relation to neuronally secreted alpha-synuclein, outlining the possible players and the mechanisms that lead to cell dysfunction and neurodegeneration (10).
Among the evidence that provide further support for a role of chronic inflammation and innate immune activation in PD is the reported increase in the serum levels in PD patients of cytokines, IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ (11). Accordingly, a strong association between markers of inflammation and risk for idiopathic PD has been reported (12). Of particular interest are Matrix metalloproteinase-3 (MMP-3) competent to degrade alpha-synuclein, which have been reported to stimulate microglia to produce pro-inflammatory and cytotoxic molecules such as TNFα, IL-6 and IL-1β as well as MMP-3, which in turn contribute to neuronal damage. MMP-3 has also been reported to damage blood-brain-barrier (BBB), and amplify neuroinflammation in an MPTP mouse model of Parkinson's disease.

Pattern Recognition Receptors

Innate immune responses are initiated by recognition of "pathogen-associated molecular patterns" (PAMPS), these are conserved structures present in infectious agents. Equally important are endogenous signals for innate responses, defined as "damage-associated molecular patterns" (DAMPS), which include nuclear and cytosolic proteins and DNA, ATP, oxidized membrane lipids, heat shock proteins (HSP) and aggregated and modified or misfolded proteins. It has been recently reported that extracellular α-synuclein may act as DAMP for microglia, increasing the expression of TLRs, MyD88, MMP-9, TNFα and IL-1β (13). The innate immunity sensors include both cell-associated "pattern recognition receptors"

(PRRs) TLRs, NOD-like receptors (NLR), RIG-like receptors (RLRs), C-type Lectin receptors, scavenger receptors and N-Formyl met-leu-phe (fMLP) receptors and soluble PRRs (14). It has been independently shown by several groups (15, 16) that alpha-synuclein, only in its amyloid fibrils form, induced inflammation through the nucleotide oligomerization domain-like receptor pyrin domain containing 3 (NLRP3) inflammasome.

Microglia, the resident immune cells

The physiological activation of microglia cells seems to protect neurons from injury and it is likely to be crucial to maintain brain's homeostasis. Coherently, after its activation microglia should return to its pre-stimulus resting state. A different picture emerges from the analysis of PD patients, in which the activation of microglia has been reported to persist leading to the reported roles for microglia and astrocytes in PD. The first damaging effect of microglia derives from the activation of NFκB pathway and consequential increase in the release of proinflammatory cytokines, such as TNF-α and IL-1β, while the release of anti-inflammatory cytokines such as IL-4, IL-13, IL-10 and TGFβ seems to be decreased. However, both pro-inflammatory and anti-inflammatory cytokines resulted elevated (17) underlining the complexity of the process of microglia activation. Anyhow, it is reasonably proved that a prolonged exposure to high levels of the proinflammatory cytokines undermines the viability of dopaminergic neurons.
A second noxious effect produced by a permanently activated microglia in PD is an uncontrolled generation of reactive oxygen species, such as NO and superoxide. These reactive species are permeable to the membrane of dopaminergic neurons, and overwhelm the capacity of the endogenous antioxidant systems to react. This process, generates a level of oxidative stress that leads to degeneration in the more vulnerable dopaminergic neurons (18).

Is immune regulation a plausible therapeutic target?

Evidence for the involvement of the immune system (both peripheral immune cells and brain resident microglia) in the development and progression of PD has inspired immunotherapeutic approaches to prevent neuronal loss. In broad terms, the approach used has been to try to hinder the signalling of microglia-derived inflammatory mediators. To control the inflammatory response one approach explored implies the use of anti-inflammatory drugs both in animal models and epidemiological studies. Their effectiveness remains controversial and a recent comprehensive study on the many trials carried out so far [(18) and table within] concludes with the not really encouraging consideration that at most (and even on this the data are no really robust) the use NSAIDs may be considered for the prevention of neurodegenerative diseases. The authors, further argue that probably these drugs result ineffective when neuronal degeneration is already in an advanced stage or detectable. Coherently, pharmacological intervention should start in the pre-symptomatic stage in order to be effective. However, this prospective

could be hindered by the absence of reliable tools for an early diagnosis. Furthermore, long-term treatment with NSAIDs may pose a number of adverse side-effects, among which gastrointestinal lesions, which could limit their long term use.

Conclusions

Based on current evidence, interventions aimed at either blocking microglia-derived inflammatory mediators or modulating the peripheral immune cells may be potentially useful therapeutic strategies that are worth exploring. The role of PD genes in modulating the immune system will hopefully unravel pathophysiologic clues that could lead to the identification of new therapeutic targets.

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This review presents an updated collection of evidence of neuroinflammation in the pathophysiology of PD but more importantly it analyses the potential role of anti-inflammatory drugs in the prevention and treatment of the disease.