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Differentiation and modulation of innate immunity response in human cord blood cells

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To my family

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ABSTRACT

Introduzione

Lo sviluppo delle barriere difensive si è rivelato un evento essenziale per l'evoluzione, in quanto ha consentito di separare l'ambiente esterno dal sempre più complesso ambiente interno. Siamo costantemente esposti ad agenti infettivi e tuttavia, nella maggior parte dei casi, siamo in grado di contrastarli, grazie al nostro sistema immunitario che agisce mediante due tipi di risposta:

- la risposta non specifica o innata, che costituisce la prima linea di difesa ed opera in modo non selettivo verso antigeni estranei;
- la risposta specifica o adattativa, diretta verso antigeni specifici.

Il tipo di risposta è dettato dalla natura dell'antigene. Una risposta immunitaria completa richiede la partecipazione coordinata di entrambe.

La risposta immunitaria innata è mediata da proteine recettoriali appartenenti alla classe dei "*pattern recognition receptors*" (PPR), che riconoscono motivi strutturali conservati presenti sugli antigeni, come componenti espresse esclusivamente da microorganismi, definite "*pathogen associated molecular patterns*" (PAMPs), o molecole rilasciate in seguito ad un danno cellulare definite "damage-associated molecular patterns" (DAMPs). In particolare, un tipo di PPR sono i "*toll-like receptors*" (TLR), una famiglia di proteine transmembrana espresse soprattutto sulla superficie delle cellule immunocompetenti, ossia monociti, macrofagi e cellule dendritiche, ma anche sulla superficie delle cellule epiteliali. L'espressione dei TLRs ha assunto un'importanza considerevole per la salute neonatale con la recente dimostrazione che i TLRs partecipano al riconoscimento di patogeni rilevanti per il neonato, tra cui Streptococco di gruppo B, *Listeria monocytogenes*, *Mycoplasma hominis*, *Candida. albicans* e *Citomegalovirus* riconosciuti dal TLR-2 o *Enterobacteriaceae, C. albicans* e il Virus Respiratorio Sinciziale dal TLR-4. Anche se l'espressione basale dei TLRs sui monociti del sangue del neonato è simile a quella degli adulti, le conseguenze funzionali della loro attivazione sono molto diverse con implicazioni in una vasta gamma di malattie, quali infezioni, immunodeficienza, sepsi, malattie autoimmuni e allergie.

Il sistema immunitario neonatale è generalmente considerato immaturo e meno funzionale rispetto alla controparte adulta e funzionalmente carente nel contrastare l'attacco di patogeni, aumentando nel neonato la suscettibilità a contrarre infezioni con conseguente incremento della mortalità. L'immaturità

del sistema immunitario neonatale deriva da effetti combinati di una serie di fattori quali l'immaturità dei suoi componenti cellulari, la mancanza di esposizione agli antigeni, l'esposizione intrauterina ad unico ambiente, che può favorire uno sviluppo di una risposta linfocitaria di tipo Th2, la bassa capacità di proliferazione dei linfociti T e l'alterata produzione di citochine Th1.
La proteina "*High mobility group box* 1" (HMGB1) è una molecola DAMP che lega il TLR-4 e il recettore RAGE ("*receptor for advanced glycation end-products*"). In principio HMGB1 è stato studiato come co-fattore nucleare coinvolto nella regolazione della trascrizione genica, ma successivamente è stato dimostrato che HMGB1 viene anche rilasciato attivamente o passivamente dalle cellule per poi agire come un citochina pro-infiammatoria. Molti studi dimostrano come HMGB1 sia implicato nella patogenesi di diverse patologie, quali artrite, cancro, epatite, malaria, ischemia del miocardio, sepsi.

Obbiettivi della ricerca

L'obbiettivo principale del lavoro è stato quello di comprendere meglio i meccanismi che sono alla base delle risposte dell'immunità innata nel neonato e nella prima infanzia per aprire la strada a possibili approcci di immunomodulazione a scopo terapeutico. In considerazione dell'importanza dei recettori TLRs nella risposta innata e della loro anomala attivazione in associazione ad alcune malattie a base allergica, è stata valutata l'associazione tra polimorfismi a singolo nucleotide (SNP) nel TRL-2 o TRL-4 e allergie atopiche in una coorte di bambini italiani allergici.
Un'altra parte dello studio è stata invece rivolta all'analisi dell'espressione di HMGB1 nel sangue del cordone ombelicale. L'espressione di HMGB1 e il suo ruolo nella risposta immunitaria sono stati studiati quasi esclusivamente nel sangue periferico (PB) di individui adulti e solo recentemente, è stato osservato che HMGB1, insieme con il recettore solubile (sRAGE), possono essere importanti mediatori del danno cellulare nel feto e fattore cruciale nella nascita pre-termine. Data l'importanza di comprendere il profilo immunitario immaturo del neonato, è stata comparata l'espressione di HMGB1 nelle cellule isolate dal sangue di cordone ombelicale (CB) rispetto al PB caratterizzando sia l'espressione di HMGB1 e la sua distribuzione nelle diverse popolazioni presenti nel sangue che valutando la sua possibile modulazione ad opera di diversi stimoli in termini di presenza nella cellula e secrezione extracellulare.

Risultati

Per studiare una possibile associazione tra polimorfismi del TLR ed allergie in pazienti in età pediatrica, SNPs R753Q nel TLR-2 o D299G nel TLR-4 sono stati individuati mediante PCR Real-Time da DNA isolato da sangue periferico. Nel gruppo di controllo, composto da 147 individui sani, il polimorfismo R753Q aveva una prevalenza del 2,5% mentre la frequenza della mutazione D299G del 12%. Nessuno dei 159 pazienti allergici ha mostrato il SNP R753Q. Nel TLR-4, invece, 7/57 pazienti affetti da allergia alimentare (12%) e 6/102 pazienti con eczema (6%) presentavano la mutazione in D299G. I dati mostrano assenza di correlazione tra i polimorfismi studiati nel TLR e allergie atopiche (eczema e allergia alimentare), suggerendo che non costituiscono marcatori per le malattie atopiche nei bambini in Italia. Recentemente, nelle malattie asmatiche, è stato riscontrato un altro fattore che sembrerebbe svolgere un ruolo importante: HMGB1. In considerazione del fatto che HMGB1, come citochina pro-infiammatoria, è stata caratterizzata esclusivamente nel sangue periferico di individui adulti, abbiamo deciso di analizzare l'espressione di HMGB1 in cellule mononucleate isolate dal CB mediante analisi in citofluorimetria (FACS). Come atteso, la totalità delle cellule permeabilizzate del CB o PB esprimevano HMGB1, in quanto HMGB1 è un cofattore nucleare ubiquitario. In assenza di permeabilizzazione, è stato possibile rilevare la presenza di HMGB1 anche sulla superficie delle cellule del CB con una percentuale del 13(±4)% (n=8), che era esattamente paragonabile a quella riscontrata sulle cellule HeLa (una linea stabilizzata da un carcinoma umano della cervice uterina che è noto esprimere HMGB1). Nel PB la proteina era presente nel 6.5(±1.8)% delle cellule (n = 8). È interessante notare che le cellule del CB presentavano un'espressione di HMGB1 più alta rispetto alle cellule del PB e tale differenza era statisticamente significativa (P=0.02).

Al fine di caratterizzare l'espressione di HMGB1 sulle differenti popolazioni cellulari presenti nel sangue, è stata effettuata una analisi citofluorimetrica utilizzando più anticorpi contemporaneamente. Nelle cellule del cordone, così come nel periferico, circa il 90% di HMGB1 era espresso su precursori delle cellule dendritiche (DCs) di tipo mieloide identificati mediante l'analisi dell'espressione di due marcatori fenotipici di membrana: $CD14^{+}CD11c^{+}$ (monociti) e $CD14^{-}CD11c^{+}$ (DCs). Solo una piccola percentuale di linfociti $CD3^{+}$ esprimeva HMGB1 [11(±8)%]. Al fine di identificare le varie sottopopolazioni linfocitarie $HMGB1^{+}$ positive, le cellule $CD3^{+}$ sono state ulteriormente caratterizzate mediante FACS utilizzando anticorpi monoclonali diretti verso il CD4, CD8 e TCR gammadelta (Vδ2). Nel CB e

PB, HMGB1 era espresso principalmente sulle cellule T γδ e $CD4^+$ mentre i linfociti $CD8^+$ sono risultati negativi. Questi esperimenti sono stati eseguiti a 48 ore dalla purificazione delle cellule mononucleate da sangue intero. Dopo 14 giorni di coltura, invece, sono state analizzate due differenti popolazioni cellulari: cellule aderenti e cellule in sospensione. Mediante analisi al FACS, è stato osservato che le cellule aderenti del CB esprimevano livelli significativamente più alti di HMGB1 rispetto alle non aderenti [14(±5)% contro il 7(± 3)% delle non-aderenti; P = 0,003]. Al contrario, nel PB le due popolazioni cellulari mostravano un livello simile di espressione. Inoltre, cellule aderenti del CB presentavano livelli di espressione di HMGB1 più elevate rispetto alla controparte del PB, in accordo con i risultati ottenuti a 48 ore, indicando che l'espressione di HMGB1 è confinata principalmente a cellule maggiormente differenziate che anche in questo caso sono state identificate al FACS come monociti e DCs.

Nel sangue periferico, la proteina HMGB1 si comporta come una citochina rilasciata dalle cellule immunitarie attivate, capace di mediare risposte a infezioni, lesioni e infiammazioni. Abbiamo così indagato se segnali diversi di attivazione, quali stimoli pro-infiammatori (TNF-α, IL-2 o IL-15) o che mimano infezioni, quali il trattamento con lipopolisaccaride batterico (LPS) o l'enterotossina B di *Staphylococcus aureus* (SEB) o il "Phorbol 12-Myristate 13-Acetate" (PMA), influenzino l'espressione di HMGB1 e la sua secrezione. L'analisi citofluorimetrica ha mostrato che tutti gli stimoli erano in grado di incrementare l'espressione di HMGB1 sulle cellule del CB e PB. Inoltre l'aumenta espressione della proteina sulla membrana cellulare era associata ad un aumento significativo dei livelli di HMGB1 rilasciati nel terreno di coltura, analizzati tramite Western Blot.

Per determinare se la modulazione dell'espressione di HMGB1 nelle cellule CB fosse associata a una sua diversa localizzazione intracellulare, cellule trattate con LPS sono state analizzate in microscopia confocale. L'immunofluorescenza ha mostrato che, in cellule del CB non trattate, HMGB1 presentava un'espressione eterogenea, localizzata principalmente nel nucleo e citoplasma. Dopo 48 ore di stimolazione con LPS, l'espressione di HMGB1 appariva principalmente sul perimetro esterno delle cellule, come indicato dalla sua co-localizzazione con la membrana plasmatica. Il coinvolgimento della membrana cellulare nella secrezione di HMGB1, osservato al FACS e in microscopia confocale, è stato ulteriormente confermato studiando l'effetto del gliburide, un inibitore del trasporto proteico di tipo non-classico. I risultati hanno mostrato che il gliburide induceva una riduzione di circa il 50% dell'espressione costitutiva di HMGB1 sulla superficie cellulare sia in CB che in PB. Inoltre, l'inibitore era

in grado di bloccare il rilascio della proteina procurato dal trattamento con LPS, ripristinando l'espressione di superficie di HMGB1 che era stata ridotta come conseguenza della sua secrezione.
Gli aminobisfosfonati (ABs) (Pamidronato o PAM, e Zoledronato o ZOL), farmaci utilizzati nell'osteoporosi e nella terapia antitumorale, sono potenti attivatori dei linfociti T γδ . Dal momento che è stato provato che HMGB1 è espresso sui linfociti T γδ, PAM e ZOL sono stati utilizzati per verificare le loro possibili capacità di modulare l'espressione di HMGB1. E' stato dimostrato che PAM e ZOL inducevano nelle cellule del CB e PB: i) l'espressione di HMGB1 sulla superficie cellulare; ii) la proliferazione di linfociti T γδ $HMGB1^+$ e iii) la secrezione di HMGB1. Per dimostrare che la secrezione di HMGB1 indotta da ABs non fosse determinata da morte cellulare, a 14 giorni di trattamento sono state valutate sia l'apoptosi che la necrosi mediante l'analisi al FACS. Non è stato osservato alcun cambiamento significativo del numero di cellule annessina V^+ e ioduro di propidio positive nel trattamento con ABs rispetto al controllo.
Avendo riscontrato che HMGB1 viene rilasciato dalle cellule a seguito di differenti stimoli, terreni pre-condizionati, generati dalla coltura di cellule del CB o PB trattate con IL-2, sono stati utilizzati per valutarne l'effetto sulla migrazione cellulare. E' stato osservato che i terreni pre-condizionati erano in grado di indurre la migrazione delle cellule monocitiche del CB e PB. Inoltre, la presenza del frammento N-terminale di HMGB1, Box A, antagonista di HMGB1 stesso, o dell'anticorpo neutralizzante anti-RAGE inibivano la migrazione di circa il 50%. Questi risultati dimostrano chiaramente che HMGB1, rilasciato dalle cellule del cordone o del sangue periferico, è funzionalmente attivo e manifesta attività chemiotattica.

Conclusioni

I TLRs hanno un ruolo fondamentale nella risposta dell'immunità innata neonatale. La perdita della loro corretta funzionalità aumenta la suscettibilità o la predisposizione di un individuo a sviluppare immunodeficienze o malattie autoimmuni. Una migliore comprensione dei meccanismi che sono alla base delle risposte dell'immunità innata neonatale potrebbe portare allo sviluppo di nuove terapie per patologie quali infezioni, cancro e allergie. Agonisti dei TLRs, per esempio, potrebbero rappresentare strumenti idonei per migliorare la difesa dell'individuo nei confronti di agenti patogeni o per ridurre potenziali allergie, modulando risposte immunitarie di tipo Th2. In realtà, diversi studi hanno indicato che alcuni polimorfismi del TLR-4 e TLR-2 sono stati associati ad allergie, quali asma o eczema atopico. In accordo con i recenti dati di letteratura, abbiamo dimostrato che, al contrario,

alcuni polimorfismi del TLR-2 e TLR-4 non sono associati con eczema e allergie alimentari nei bambini italiani allergici, a indicare che la correlazione tra la malattia e il polimorfismo del TLR potrebbe essere influenzata positivamente o negativamente da fattori diversi quali il corredo genetico di ogni singolo individuo, la natura degli antigeni associati o l'ambiente in cui vive.
Recentemente, nel fluido di lavaggio bronco-alveolare di pazienti con malattia polmonare ostruttiva cronica sono stati osservati elevati livelli di HMGB1 che è stato considerato, insieme al suo recettore solubile sRAGE, un nuovo biomarker nell'asma grave. Questi dati suggeriscono che HMGB1 potrebbe avere un ruolo nelle malattie asmatiche. Considerando che l'asma e le allergie atopiche sono considerate malattie infiammatorie, inibire il rilascio extracellulare di HMGB1 potrebbe rappresentare un trattamento terapeutico idoneo per il trattamento di queste patologie.
E' anche stata messa in luce l'importanza di HMGB1 come mediatore di infiammazione, nel sistema neonatale oltre che nell'adulto. Infatti, il lavoro svolto nel corso del dottorato ha messo in evidenza, per la prima volta, che cellule mononucleate isolate dal sangue umano del cordone ombelicale esprimono e rilasciano HMGB1. HMGB1 è presente principalmente su una popolazione di cellule differenziate, quali DCs e monociti, e in misura minore su i linfociti T CD4 e $\gamma\,\delta$. Questi risultati sono completamente in accordo con il ruolo che HMGB1 ha nella risposta immunitaria innata, che vede macrofagi attivati, monociti, DCs e linfociti T $\gamma\,\delta$ come principali attori. Inoltre, è stato rilevato che stimoli diversi, quali stimoli pro-infiammatori o antigeni dei linfociti T $\gamma\,\delta$, come Pamidronato e Zoledronato, modulano l'espressione di HMGB1 e la sua secrezione secondo la via di secrezione non classica. Questo studio fornisce la prima dimostrazione che gli aminobifosfonati sono in grado di modulare l'espressione di HMGB1 in cellule del CB e del PB, coinvolgendo linfociti T $\gamma\,\delta$ direttamente o attraverso le cellule APC (*antigen presenting cells*). HMGB1, rilasciato nell'ambiente extracellulare, può funzionare come citochina ed esprimere capacità chemottatiche verso i monociti. L'identificazione di molecole capaci di inibire l'attività di HMGB1 sta assumendo notevole interesse clinico. Alcuni studi hanno dimostrato la fattibilità dello sviluppo di modulatori di HMGB1 per nuove terapie, sistemiche e locali, che hanno come bersaglio patologie infiammatorie. La recente identificazione della funzione inibitrice della glicirrizina ha portato sul mercato italiano alla commercializzazione di uno spray nasale per la terapia della rinite e della poliposi.
Inibire il rilascio extracellulare di HMGB1 potrebbe rappresentare una strategia terapeutica idonea per il trattamento dell'infiammazione, mentre

indurne il rilascio potrebbe permettere lo sviluppo di una risposta immunitaria cellulo-mediata di tipo Th1 essenziale per un'ottima immunizzazione a seguito di vaccinazione. Per immunoterapia biologica si intende un trattamento basato sull'uso e/o la modulazione di componenti del sistema immunitario per promuovere una risposta immunitaria efficace contro le malattie. E' stato dimostrato che HMGB1 extracellulare funziona come immuno-adiuvante ad esempio aumentando l'immunogenicità di cellule di linfoma oppure ottimizzando la risposta anticorpale alla proteina solubile ovalbumina. Inoltre, Hp91, un corto frammento peptidico della proteina HMGB1, induce attivazione di DCs, aumentando la secrezione di citochine pro-infiammatorie di tipo Th1 e di chemochine. Pertanto, HMGB1 viene proposto come nuovo adiuvante per vaccini. In questo scenario, gli ABs, che sono in grado di stimolare l'immunità innata, tramite l'attivazione di linfociti γδ e la secrezione di HMGB1, potrebbero essere presi in considerazione come immuno-modulanti per patologie neonatali. Considerando che ZOL o PAM sono utilizzati nella terapia antitumorale, gli ABs potrebbero interferire nella complessa interazione tra tumore e sistema immunitario dell'ospite tramite il rilascio di mediatori infiammatori, come HMGB1, che mediano la presentazione di antigeni tumorali e l'induzione di una risposta antigene tumorale-specifica dei linfociti T citotossici. La capacità immuno-adiuvante di HMGB1 rende questa proteina un candidato promettente anche nell'immunoterapia dei tumori.

INTRODUCTION

IMMUNITY

We are constantly being exposed to infectious agents and yet, in most cases, we are able to resist them. It is our immune system that enables us to resist infections. The immune system is composed of two major subdivisions, the innate or non-specific immune system and the adaptive or specific immune system. The innate immune system is our first line of defense against invading organisms while the adaptive immune system acts as a second line of defense and also affords protection against re-exposure to the same pathogen. Each of the major subdivisions of the immune system has both cellular and humoral components by which they carry out their protective function. In addition, the innate immune system also has anatomical features that function as barriers to infection. Although these two arms of the immune system have distinct functions, there is interplay between these systems (i.e., components of the innate immune system influence the adaptive immune system and vice versa).

Although the innate and adaptive immune systems function to protect against invading organisms, they differ in a number of ways. The adaptive immune system requires some time to react to an invading organism, whereas the innate immune system includes defenses that, for the most part, are constitutively present and ready to be mobilized upon infection. Second, the adaptive immune system is antigen specific and reacts only with the organism that induced the response. In contrast, the innate system is not antigen specific and reacts equally well to a variety of organisms. Finally, the adaptive immune system demonstrates immunological memory. It "remembers" that it has encountered an invading organism and reacts more rapidly on subsequent exposure to the same organism. In contrast, the innate immune system does not demonstrate immunological memory.

The main function of the immune system is self/non-self discrimination. This ability to distinguish between self and non-self is necessary to protect the organism from invading pathogens and to eliminate modified or altered cells (e.g. malignant cells). Since pathogens may replicate intracellularly (viruses and some bacteria and parasites) or extracellularly (most bacteria, fungi and parasites), different components of the immune system have evolved to protect against these different types of pathogens. It is important to remember that infection with an organism does not necessarily mean diseases, since the immune system in most cases will be able to eliminate the infection before

disease occurs. Disease occurs only when the bolus of infection is high, when the virulence of the invading organism is great or when immunity is compromised or immature, as in umbilical cord blood. Although the immune system, for the most part, has beneficial effects, there can be detrimental effects as well. During inflammation, which is the response to an invading organism, there may be local discomfort and collateral damage to healthy tissue as a result of the toxic products produced by the immune response. In addition, in some cases the immune response can be directed toward self tissues resulting in autoimmune disease.

Innate immunity

Broadly defined, the innate immune system includes all aspects of the host's immune defense mechanisms that are encoded in their mature functional forms by the germ-line genes of the host. These include physical barriers, such as epithelial cell layers that express tight cell-cell contacts (tight junctions, cadherin-mediated cell interactions, and others) or the secreted mucus layer that overlays the epithelium in the respiratory, gastrointestinal and genitourinary tracts. To protect the body from attack extraneous agents and to mount an immune response, cellular components are essential: i) with phagocytic activity (neutrophilis and monocytes/macrophages); ii) with cytotoxic activity Natural (Natural Killer or NK); iii) dendritic cells and γδ T cells.

Soluble proteins and bioactive small molecules that are either constitutively present in biological fluids (such as the complement proteins, defensins, and ficolins1–3) or that are released from cells as they are activated (including cytokines that regulate the function of other cells, chemokines that attract inflammatory leukocytes, lipid mediators of inflammation, reactive free radical species, and bioactive amines and enzymes that also contribute to tissue inflammation) are also involved in innate response.

Several different receptors, called pattern-recognition receptors (PRRs) [**1**] are used by the innate immune system to recognize and signal presence of pathogens. This recognition can lead to different events, such as stimulation of phagocytosis when the macrophage mannose receptor is engaged. Signal through the evolutionary conserved Toll-like receptor (TLRs) can upregulate co-stimulatory molecules on macrophages and dendritic cells, enabling these to initiate an adaptive immune response. Thus TLRs are an important bridge between innate and adaptive immunity. Unlike adaptive immunity, innate immunity is programmed to recognize series of molecular patterns present at the infected lesion: i) the patterns that are presented by microorganisms [pathogen-associated molecular patterns (PAMPs)], and (ii) the patterns of

host intracellular molecules secreted by dying host cells into the extracellular spaces upon microorganism-induced damage [damage-associated molecular patterns (DAMPs)] [**2-5**]. Thus, the emergence of PAMPs and DAMPs together signals of invasion by pathogenic microorganisms are closely associated to tissue damage. Thus, the emergence of PAMPs and DAMPs together signals of invasion by pathogenic microorganisms are closely associated to tissue damage. TLRs represent a key molecular link between tissue injury, infection, and inflammation.

Innate immunity of newborn

All organ systems of the body undergo a dramatic transition at birth, from a sheltered intra-uterine existence to the radically distinct environment of the outside world. This acute transition is then followed by a gradual, age-dependent maturation. The fetal and neonatal immune systems are associated with physiological demands that are: protection against infection, including viral and bacterial pathogens at the maternal–fetal interface [**6, 7**]; avoidance of potentially harmful pro-inflammatory/T helper 1 (Th1)-cell polarizing responses that can induce alloimmune reactions between mother and fetus [**8**], and mediation of the transition between the normally sterile intra-uterine environment to the foreign antigen-rich environment of the outside world, including primary colonization of the skin and intestinal tract by microorganisms. Given the limited exposure to antigens in utero and the well-described defects in neonatal adaptive immunity [**9**], newborns must rely on their innate immune systems for protection to a significant extent [**10, 11**]. The neonatal immune system is generally considered to be immature and less functional compared to adult counterpart [**6**]. In fact the impairment of the newborn immune system may result from the combined effects of a number of factors as: immaturity of its cellular components; lack of previous exposure to antigens; intra-uterine exposure to unique hormonal and cytokine environment which may favor Th2 subset development; low proliferation capacity of T lymphocytes and its impaired Th1 cytokine production. This immaturity is thought to account for the failure of the newborn to mount robust and protective response against several pathogens, resulting thus in increased mortality [**12, 13**]. However there is still controversy about the factors underlying this hypo-responsiveness.

The potential relevance of TLR expression in neonatal health and disease includes the recent demonstration that TLRs participate in the recognition of microbial pathogens that are relevant to neonates, including pathogens recognized by TLR2 (group B Streptococcus, Listeria monocytogenes, Mycoplasma hominis, C. albicans hyphae and cytomegalovirus) or TLR4

(Enterobactericeae, C. albicans blastoconidia1and respiratory syncitial virus). Although basal TLR expression of full-term neonatal blood monocytes is similar to that of adults, the functional consequences of neonatal TLR activation are very different. It has been appreciated for some time that, despite the presence of higher concentrations of monocytes at birth, the addition of LPS to whole cord blood from human newborns results in diminished production of TNF compared with adult peripheral blood [**14**].
In view of importance to comprehend immature and innate immune profiles of newborns, numerous studied are performed in umbilical cord blood (or cord blood, CB) in comparison to adult counterpart (PB). Several authors have reported that CB is characterized by a phenotypically and functionally immature immune system [**15**]. Therefore, in comparison to mononuclear cells of PB, CB shows a reduced production of cytokines such as IL-2, IL-3, IL-4, the factor stimulating colony formation of granulocyte (G-CSFs), IL-13, the factor stimulating the formation of colonies of macrophages (M-CSF), transforming growth factor β1 (TGF-β1), IL-12, IL-15, IL-18, TNF and IFNγ and granule-specific molecules, such as lipase or perforin [**16**, **17**]. Moreover, T lymphocytes and NK cells of CB possess a lower reactivity than adult cells, indicating a lower proliferative capacity in response to alloantigens [**18, 19**]. Anyway, the functional role of these cells into the neonatal immune response is not clear.

γδ T lymphocytes

γδ T cells (gamma delta T cells) represent a small subset of T cells that possess a distinct T cell receptor (TCR) on their surface. A majority of T cells have a TCR composed of two glycoprotein chains called α- and β- TCR chains. In contrast, in γδ T cells, the TCR is made up of one γ-chain and one δ-chain. This group of T cells is usually much less common than αβ T cells, but are found at their highest abundance in the gut mucosa, within a population of lymphocytes known as intraepithelial lymphocytes (IELs).
The antigenic molecules that activate γδ T cells are still largely unknown and current concepts of γδ T cells as 'first line of defense', 'regulatory cells', or 'bridge between innate and adaptive responses' [**20**].
γδ T cells, specifically the Vγ9/Vδ2 subset, are unique to humans and primates and represent a minor and unconventional constituent of the leukocyte population in PB (5-10%) and CB (1-3%). They are assumed to play an early and essential role in sensing 'danger' by invading pathogens as they expand dramatically in many acute infections and may exceed all other lymphocytes within a few days, e.g. in tuberculosis, salmonellosis, ehrlichiosis, brucellosis, tularemia, listeriosis, toxoplasmosis and malaria.

γδ T cells are peculiar in that they do not seem to require antigen processing and MHC presentation of peptide epitopes and recognize in a TCR-dependent fashion a restricted set of phosphorylated compounds referred to as "phospho-antigens" (PhAgs), which are produced through the isoprenoid biosynthetic pathway [**21-23**]. The discover and identification of γδ T cell specific antigens started with the observation that Vγ9Vδ2 T cells are reactive against extract from *Mycobacterium tuberculosis (Mtb)* [**24-26**]. The initial antigens from Mtb were shown to be small, soluble, non-peptidic, phosphorylated compounds [**25, 27**]. A number of γδ T cell antigens have been identified, mainly anionic molecules that invariably contain a phosphate moiety. Aminobisphosphonate (ABs) are synthetic compounds [**28**], known as potent inhibitors of osteoclast-mediated bone resorption used for the treatment of osteoporosis, bone metastasis and cancer [**29-32**]. It has been shown that bisphosphonates exert a stimulatory effect on adult PB γδ T cells, in vitro and in vivo, by inhibiting the mevalonate pathway [**21, 22, 33**] Considering that CB Vγ9Vδ2 T cells are considered to be immature because they have naïve phenotypes and display poor proliferative [**34**] or cytokine responses [**35**], recently, we have reported that the treatment with ABs induces proliferative responses in cord blood Vδ2 T cells accompanied by modifications their naïve phenotype towards a regulatory subset, indicating that they are not inherently unresponsive [**36, 37**].

It's already known that Vγ9Vδ2 T cell activation play a wide immunological role in the orchestration of the immune response. They are able to directly inhibit viral replication both through cytolitic and non-cytolitic mechanisms and, on the other hand, Vγ9Vδ2 T cells induce the activation or differentiation of other immune cells. Specifically, they can drive Th1 polarization, DCs differentiation and B cell activation [**38, 39**]. Extensive studies were performed on DC-Vγ9Vδ2 T cells interaction. PhAgs-activated γδ T cells induce the maturation of DCs by inducing the expression of costimulatory markers, MHC molecules and chemokine receptors for homing in the lymphoid organs, suggesting that Vγ9Vδ2 T cell activation cooperate in the induction of adaptive response. On the other hand, DCs promote γδ T cell activation resulting in the expression of high levels of CD69 and production of pro-inflammatory cytokines such as TNF-α and IFN-γ, suggesting a reciprocal interaction and a positive feedback [**40**]. Most studies are focused on understanding of immunology of Vγ9Vδ2 T cell population isolated from PB, while still little is known about the γδ T cells isolated from human umbilical cord blood.

TLRs

TLRs are the most widely studied PRRs and are considered to be the primary sensors of pathogens. The field of TLR immunobiology expanded rapidly after the discovery of toll proteins in flies [**41**]. In humans, 10 TLR family members have been identified (there are 12 in mice). TLRs are type I membrane glycoproteins and consist of extracellular leucine rich repeats (LRRs) that are required for ligand recognition, and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain, required for downstream signaling. The crystal structure of the extracellular recognition domain of several TLRs bound to their agonist or antagonist PAMPs has been characterized. TLRs have a unique horseshoe, or "m" shaped architecture [**42, 43**]. The intracellular domain is required for the interaction and recruitment of various adaptor molecules to activate the downstream signaling pathway [**44, 45**]. TLRs are expressed on various immune cells, including DCs, macrophages, and B cells, but its expression can vary depending on the activation status or the cell subset. TLR expression has been even identified on non-immune cells, such as fibroblasts and epithelial cells [**46, 47**]. To date, 11 TLRs in humans and 13 TLRs in mice have been identified, with each receptor recognizing distinct PAMPs derived from various pathogens, including bacteria, viruses, protozoa, and fungi. TLRs are expressed in distinct cellular compartments: TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 (only found in mice) are expressed on the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 are located in the endosome.

TLR ligands

TLRs can be classified into several groups based on the types of PAMPs - also known as TLR ligands - they recognize (Table I).

Table I PRRs and Their Ligands

PRRs	Localization	Ligand	Origin of the Ligand
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma	Lipoprotein	Bacteria, viruses,

PRRs	Localization	Ligand	Origin of the Ligand
	membrane		parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa

TLRs sense mainly components of the bacterial cell wall and nucleic acids expressed by microbes. TLR1, 2, 4, 5 and 6 are primarily expressed on the cell surface and recognize PAMPs derived from bacteria, fungi and protozoa, whereas TLR3, 7, 8 and 9 are exclusively expressed within endocytic compartments and primarily recognize nucleic acid PAMPs derived from various viruses and bacteria [**48**, **49**]. Upon ligation, TLRs dimerize to homo- or heterodimers. TLR4, together with its coreceptors MD-2 and CD14, recognizes lipopolysaccharide (LPS) from gram-negative bacteria [**50**, **51**]. TLR2 forms heterodimers with TLR1, TLR6, and non-TLRs such as CD36 to discriminate a wide variety of TLR ligands, including peptidoglycan, lipopeptides, and lipoproteins of gram-positive bacteria, mycoplasma

lipopeptides and fungal zymosan. In particular, TLR1/2 and TLR2/6 are able to discriminate triacyl- and diacyl-lipopeptide, respectively [**52**]. Flagellin from flagellated bacteria is recognized by TLR5 [**53**], whereas mouse TLR11 senses yet unknown structures of uropathogenic bacteria [**54**] and profilin-like protein of the protozoan parasite Toxoplasma gondii [**55**].

Intracellular TLRs, expressed in the endosome, are involved in the recognition of bacterial and viral-derived nucleic acids. TLR3 recognizes double-stranded RNA (dsRNA), which is generated during replication of many viruses. PolyI:C is a synthtetic ligand of TLR3 [**56**]. TLR7 senses synthetic imidazoquinoline-like molecules, guanosine analogues such as loxoribine, single-stranded RNA (ssRNA), and small interfering RNA (siRNA) [**57**]. Human TLR8, with highest homology to TLR7, participates in the detection of imidazoquinolines and ssRNA, whereas in mice the function and ligands of TLR8 remain elusive. TLR9 is responsible for the recognition of CpG-DNA motifs present in bacterial and viral genomes [**58**].

TLRs as PRRs are critically involved in the discrimination between "self" and "non-self". In the last decade a number of endogenous molecules specifically generated upon tissue injury, DAMPs, activate TLRs, especially TLR4, TLR7/8, and TLR9. Some are intracellular molecules normally inaccessible to the immune system that are released into the extracellular milieu as a result of cell necrosis or activation following injury, including high mobility group box 1 (HMGB1), heat shock proteins, interleukin-1α (IL-1α), defensins, annexins, and S100 [**59-63**] (Fig. 1). Others are extracellular matrix (ECM) molecule fragments that are released upon tissue damage or ECM molecules that are specifically upregulated in response to tissue injury [**64**]. DAMPs are key danger signals that alert the organism to tissue damage and initiate the process of tissue repair.

According to the theory of the "danger" model postulated by Polly Matzinger [**4, 65**], the immune system does not solely tend to discriminate between "self" and "foreign", but is rather activated by "danger" signals derived from damaged and stressed tissue. Thus, stimulation of TLRs by endogenous ligands may contribute to the pathogenesis of many inflammatory and autoimmune diseases.

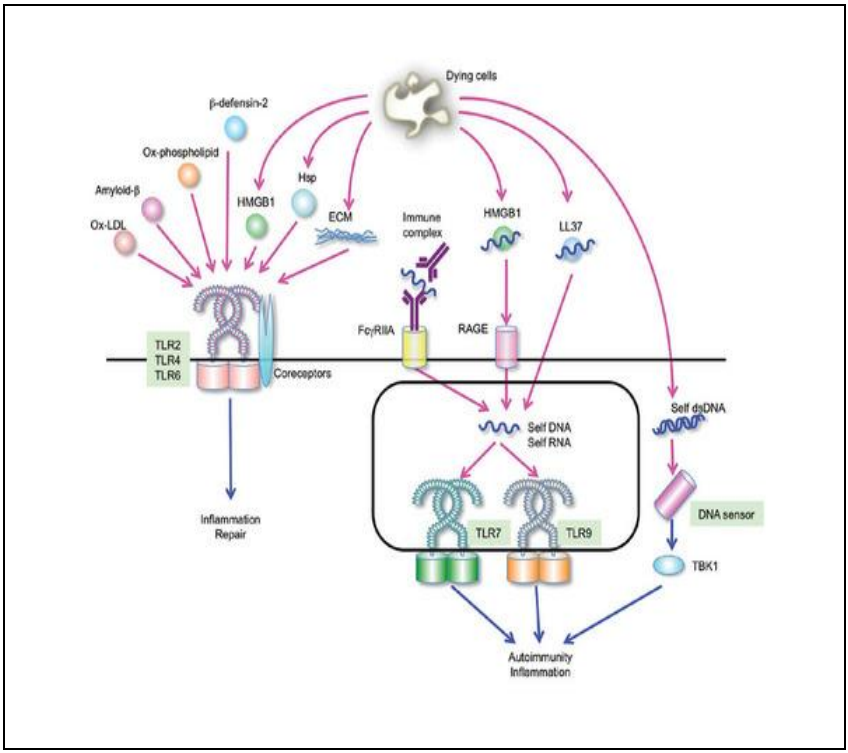

FIG 1. DAMPs. Endogenous molecules released by dying cells, such as HMGB1, heat-shock proteins (Hsp) and ECM components, are recognized by TLR2, TLR4 or TLR2-TLR4. Amyloid-β and oxidized LDL (Ox-LDL) are both sensed by TLR4-TLR6 along with the coreceptor CD36. Oxidized (Ox-) phospholipids generated after infection and the antimicrobial peptide β-defensin 2 are recognized by TLR4. Recognition of these endogenous molecules by cell surface TLRs leads to inflammation as well as repair responses. Self DNA and RNA in complex with LL37 are internalized into early endosomes and are recognized by TLR9 and TLR7, respectively. The HMGB1–self DNA complex is internalized via RAGE and is recognized by TLR9. Immune complexes containing self nucleic acids are internalized via Fc receptors, such as FcγRIIa, and stimulate TLR7 and TLR9. Self DNA incompletely digested during apoptosis is probably sensed by an intracellular DNA sensor that activates TBK1. The recognition of self nucleic acids by TLR7, TLR9 and an as-yet-undefined DNA sensor leads to the induction of type I interferon and promotes autoimmune and/or inflammatory diseases [**5**].

TLRs and diseases

Dysfunction of TLRs is implicated in a wide range of human diseases, especially in infection, immunodeficiency, sepsis, autoimmune disorders and allergy. TLRs recognize a wide variety of putative host-derived agonists that have emerged as key mediators of innate immunity. TLR signaling also plays an important role in the activation of the adaptive immune system by inducing pro-inflammatory cytokines and up-regulating costimulatory molecules of antigen presenting cells (APCs). Inappropriate activation of TLRs by endogenous ligands released by damaged tissues may result in sterile inflammation. Sepsis, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), type I diabetes, and multiple sclerosis (MS) are characterized by aberrant TLR activation. Aberrant TLR activation is also thought to contribute to cancer and atherosclerosis (reviewed in [**66- 70**]).

In spite of the protective effects of TLRs upon infection, faulty TLR signaling and polymorphisms in the TLR genes are increasingly implicated in the pathogenesis of allergic diseases. One explanation has been related to the so called hygiene hypothesis. This hypothesis states that a lack of early childhood exposure to infectious agents, symbiotic microorganisms (e.g. gut flora) and parasites increases the susceptibility to allergic diseases by modulating the development of the immune system. During normal circumstances, infectious stimuli (via TLRs) lead to Th1-mediated responses. A reduction in TLR activation reduces the Th1 responses, resulting in unrestrained Th2-mediated immunity that is associated with atopy. This might explain the increase in allergic diseases seen in the western world during the last decades. Actually, several studies have indicated that polymorphisms or the impaired signalings of TLRs were correlated with a increased risk for allergy in adults or children. TLR-4 D299G and TLR-2 R753Q polymorphisms have been associated with asthma or atopic eczema [**71-73**] and its defective signaling led to allergic sensitization to food protein in mice [**74, 75**]. High risk newborns for allergy have also been noted to have altered generation of putative regulatory T-cell populations after LPS stimulation, presumably through TLR-4 pathways [**72**]. Also TLR-2 mutation has been associated with a higher risk for asthma in European children [**73**] and with atopic dermatitis having severe phenotype [**76, 77**]

Hence, in recent years TLRs and associated signalling molecules have become attractive targets for the development of new drugs, as adjuvants for existing and new vaccines.

HMGB1

High mobility group box 1 (HMGB1) was described over three decades ago as nuclear protein. The protein was given its name because of its ability to migrate rapidly in agarose gels during electrophoresis [**78**]. HMGB1 is an abundant protein and is distributed in all mammalian nucleated cells. More than one million molecules per nucleus can be found in the thymus [**79**]. Intracellulary, HMGB1 is more concentrated in the cytoplasm of cells in the cytoplasm of cells in the liver and brain and is concentrated in the nuclei of most other tissue [**80**].

Over the years HMGB1 has been studied and additional properties besides its originally described nuclear functions have been revealed. Extracellular HMGB1 induces migration, recruits stem cells, possesses antibacterial functions and complexed HMGB1 induces cytokine production. In this section the different properties of HMGB1 will be discussed.

Structure

HMGB1 is highly conserved between species with a sequence omology of 99% between the rodent and human forms, and is present in all mammalian tissues. HMGB is a family of three nuclear proteins including HMGB1 (previously named amphoterin or HMG1), HMGB2 (previously named HMG2), and HMGB3 (previously named HMG4 or HMG2b) [**81**]. HMGB1 is 215 amino acids in length with two domains composed of 80 amino acids referred to as " HMG boxes A and B". The "A" and "B" box of the protein interact with DNA and lead to distortion and bending of the double helix [**82**] (Fig. 2). It facilitates the binding of several regulatory protein complexes to DNA, facilitates the integration of transposons, such as the Sleeping Beauty transposon [**83**] and enhances the interaction of other proteins with DNA, as p53, NF-κB, homeobox-containing proteins, steroid hormone receptors and recombination activating gene 1/2 (RAG1/2) proteins which are needed for VDJ recombination in T and B lymphocytes [**84-86**] .

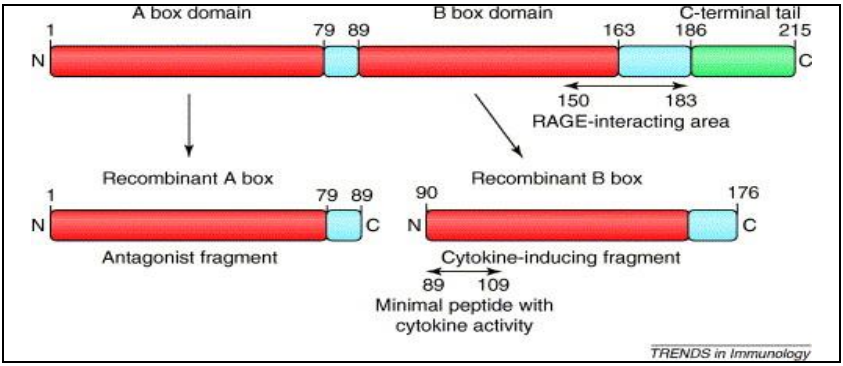

FIG. 2. **Representation of HMGB1**. HMGB1 is a 30 kD nuclear protein of 215 amino acids. It comprises two DNA-binding domains (red): the A box and the B box, and a negatively charged C-terminal tail (green). The blue boxes do not encode functional domains. Truncation of HMGB1 demonstrates that the recombinant A box (1–89) acts as a specific antagonist, whereas the cytokine activity of HMGB1 is produced by the recombinant B box (90–176). The first 20 amino acids of the recombinant B box represent the minimal peptide that maintains cytokine activity. The region involved in the interaction of HMGB1 with the receptor for advanced glycation end-products (RAGE) is located between residues 150 and 183 [**87**].

Extracellular release

HMGB1 can be secreted from cells in two ways, either passively or actively (Fig. 3). Necrotic cells release their HMGB1 passively whereas, according to the previous observations, cells undergoing apoptotic cell death would not release HMGB1 due to sequestration of HMGB1 to the condensed chromatin [**88, 89**]. However, it has recently been demonstrated that certain apoptotic cells undergoing secondary necrosis and any passively leak HMGB1, indicating that the originally described dichotomy between necrosis and apoptosis may be not actually be so distinct [**90**].

In response to pro-inflammatory stimuli such as LPS, IFN-γ, INF-α/β, and nitric oxide, HMGB1 can actively be released from a number of different cell types including macrophages, pituicytes, mature dendritic cells, NK cells and fibroblasts [**91, 92**] and protein synthesis is not required [**93**]. HMGB1 associates and dissociates rapidly from the chromatin in living cells and continually traffics between the nucleus and cytosol [**94**]. Following exposure to inflammatory stimuli, HMGB1 relocates from the nucleus to the cytosol, where acetylation of its lysine residues blocks its import into the nucleus. HMGB1 lacks a secretory signal sequence and is not routed through the endoplasmic reticulum and the Golgi apparatus [**93, 95**]. It is instead

packaged into secretory lysosomes, a specific population of lysosomes present in haematopoietic cells [**96**] before being released extracellularly.
The mechanism underlying the active secretion is incompletely understood. Studies demonstrate that HMGB1 release is mediated via a non-classical pathway by Ca2+ regulated endose-like organelles termed secretory lysosomes [**93**]. Secretory lysosomes are expressed in hemopoietic cells which do play an important role in immune and inflammatory events. The role of transporters in non-classical protein secretion has been widely studied. One suggested transporter for HMGB1 is the ATP-binding cassette transporter [**93**]. Inhibition of this transporter inhibits HMGB1 release from monocytes /macrophages [**97**].

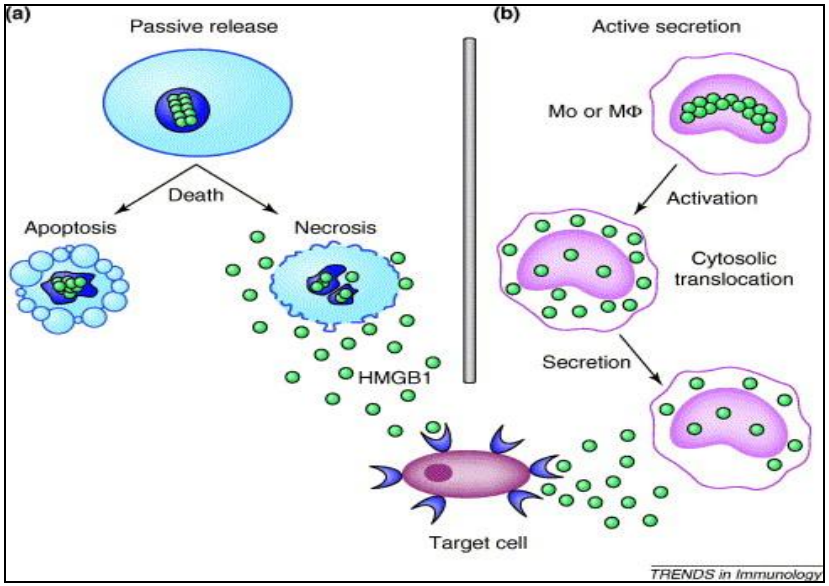

FIG. 3 Release of HMGB1 within the extracellular space. In most cells, HMGB1 (green) is a mobile nuclear protein that constantly shuttles between the nucleus and the cytosol. There are two situations in which HMGB1 reaches the extracellular environment: (a) passive release from necrotic cells or (b) active secretion from cells of the innate immune system. Necrotic cells have leaky plasma membranes and HMGB1 diffuses into the surrounding milieu. By contrast, apoptotic cells retain HMGB1 bound to chromatin. Active secretion of HMGB1 from monocytes (Mo) or macrophages (MΦ) occurs following activation by microbial and proinflammatory stimuli. This leads to acetylation of specific lysine residues, which blocks the import of HMGB1 into the nucleus. Cytosolic HMGB1 is then packaged in secretory vesicles before being released into the environment. The effects induced by extracellular HMGB1 vary with the target cells and are mediated by binding to membrane receptors [**87**].

Receptors

The first described receptor for HMGB1 was the receptor for advanced glycation end products (RAGE) [**98**]. However, since RAGE-deficient cells were shown to still be able to respond to HMGB1 stimulation and anti-rage antibodies only partially suppressed the activity of HMGB1, RAGE is not believed to be the only receptor for HMGB1 [**99**]. Recently, HMGB1 has been described to be a ligand of toll-like receptors (TLR) 2 and 4 [**100**].

RAGE belongs to the immunoglobulin (Ig) superfamiles and comprises of three extracellular Ig domains, a single transmembrane segment and short cytoplasmic tail. It interacts with other structurally unrelated ligands including several members of the S100 family, amyloid-beta peptide, transthyretin and beta2 integrin Mac-1.

RAGE signals through pathways that involve ERK1 (extracellular-signal-regulated kinase) and/or ERK2, and the mitogen-activated protein kinase p38, and it promotes the activation of nuclear factor-κB (NF-κB). In a MyD88 (myeloid differentiation primary-response protein 88)-dependent manner, high-mobility group box 1 protein (HMGB1)-mediated signalling leads to activation of the IKK complex (inhibitor of NF-κB (IκB) kinase complex), consisting of IKK-α,IKK-β and NF-κB essential modulator (NEMO), which phosphorylates IκBα and thereby releases NF-κB for translocation to the nucleus and allows the transcription of pro-inflammatory genes (such as interleukin-1 (IL-1), IL-6 and tumour-necrosis factor).

Interaction of HMGB1 with TLR2 and TLR4 might therefore enable HMGB1 to promote inflammatory responses that are similar to those of lipopolysaccharide. The common signalling pathway for RAGE, TLR2 and TLR4 involves a MyD88-dependent pathway that ultimately leads to NF-κB activation. Little is known about the precise means by which phospho-ERK and p38 are activated by HMGB1, but these molecules, together with stress-activated protein kinase (SAPK) and JUN aminoterminal kinase (JNK), are activated within several minutes, even in the absence of expression of RAGE or the IL-1 receptor α-chain137 (Fig. 4).

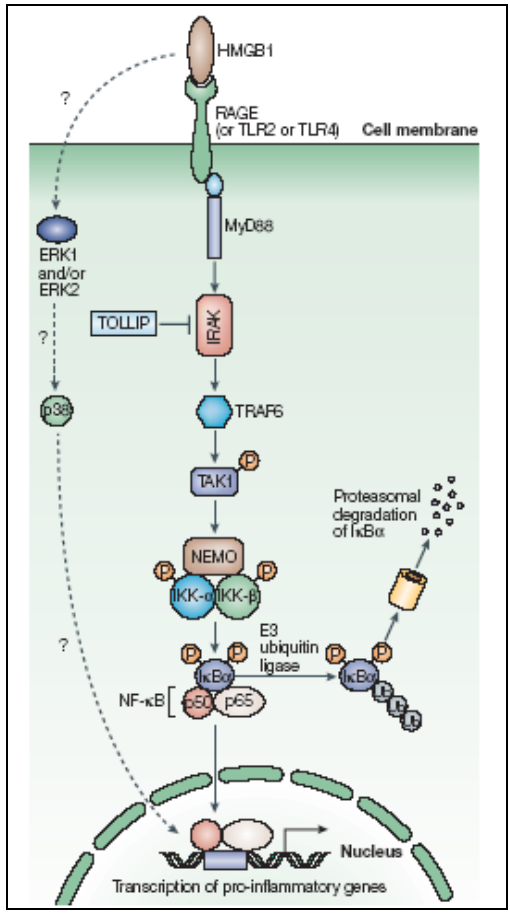

FIG. 4 Signalling pathways downstream of RAGE, TLR2 and TLR4 that mediate the effects of HMGB1 [**101**].

HMGB1 as cytokine

In 1999, during a course of experiments designed to identify late-acting mediators of endotoxaemia and sepsis, Wang and colleagues discovered that activated macrophages secrete HMGB1 as a delayed mediator of inflammation [**91**]. This HMGB1 release occurs considerably later than

secretion of the classical early pro-inflammatory mediators TNF and IL-1. In a standardized model in which mice are administered lipopolysaccharide (LPS) to generate endotoxaemia, serum HMGB1 levels begin to increase 12–18 hours after peak levels of TNF, which occur at 2 hours, and of IL-1, which occur at 4–6 hours [**102, 103**]. Administration of HMGB1-specific antibodies confers significant protection against the lethal effects of endotoxin, even when antibody dosing is delayed until after the peak levels of TNF and IL-1. HMGB1 is now recognized as a cytokine because it mediates systemic inflammatory responses, is secreted by activated immune cells, activates prototypical inflammatory responses in immune cells and endothelial cells. HMGB1 stimulation of PBMC led to release of TNF, IL-1a, IL-1b, IL-6, IL-8 macrophage inflammatory protein (MIP)-1a and (MIP)-1b. Moreover, a dose-dependent increase in ICAM, VCAM and RAGE expression on endothelial cells following HMGB1 stimulation has also been observed [**104, 105**].

HMGB1 is one of several DAMPs (including heat-shock proteins, uric acid, ATP and S100 molecules) that facilitate the recruitment and activation of macrophages, plasmacytoid DCs (pDCs) and myeloid DCs, thereby promoting inflammation and/or tissue repair. Activated NK cells (which accumulate in response to HMGB1 and other pro-inflammatory signals) provide an additional source of HMGB1, which is released into the immunological synapse between NK cells and immature DCs and promotes the maturation of DCs and the induction of T-helper-1-cell responses [**90, 106, 107**]. More speculative is the ability of mature DCs to produce HMGB1 and therefore stimulate mature T cells. HMGB1 might also have a role in inhibiting the IFN-γ response of pDCs to CpG-containing DNA. In the lymphnode, mature DCs not only provide both antigen in the form of peptide–MHC complexes and co-stimulatory molecules, but also are a source of HMGB1, which matures additional DCs that are recruited across high endothelial venules (HEVs), enabling further interaction with, and stimulation of, naive CD4+ and CD8+ T cells.

However recent studies indicate that highly purified HMGB1 may not by itself be active as a pro-inflammatory mediator. Several recent reports indicate that HMGB1 needs to form a complex with pro-inflammatory ligands, as LPS, CpG-DNA or IL-1β, to exert its synergistic influence [**108, 109**].

Table II Biological function of HMGB1 and its target cells

Biological activity	Target cells
Secretion of pro-inflammatory factors Promotes transendothelial migrations of monocites	Monocytes and macrophages, DCs
Increased expression of genes for pro-inflammatory factors	Neutrophils
Increased immunogenicity of soluble or corpusculate antigens	DCs
Maturation of DCs and Th1 polarization	DCs
Upregulation of adhesion molecules	Endothelial cells
Chemotaxis Promotes differentiation	Stem cells
Cytoskeleton reorganization and transendothelial migration	Monocytes, vascular smooth muscle cells, vessel-associated stem cells (mesangioblasts)
Proliferation	Vessel-associated stem cells (mesangioblasts)
Enhances Invasiveness	Tumor cells

Diseases

Several studies implicate HMGB1 in the pathogenesis of various inflammatory conditions and diseases, as arthritis, cancer, hepatitis, malaria, myocardial ischemia, sepsis.

The first evidence that links HMGB1 to sepsis was obtained more than ten years ago when, in a pioneering study, HMGB1 was identified as a late mediator of lethal systemic inflammation and as being involved in the delayed lethality of endotoxin and systemic inflammation [**91**].

Since then, HMGB1 has been an increasingly attractive target for drug development because considerable data has been generated on its role in both acute and chronic inflammatory diseases. Preclinical studies have validated the possibility of targeting HMGB1 as a therapeutic agent, by using independent approaches [**110**], including anti-HMGB1 antibodies and the A box fragment of HMGB1, which has antagonistic actions. Recently, encouraging results have been obtained, including the blocking of RAGE-HMGB1 signaling [**111**]. The identification of HMGB1 polymorphisms as significant factors associated with early and late mortality systemic inflammatory response syndrome and sepsis hints at a possible role for HMGB1 genetics in predictive medicine [**112, 113**]. HMGB1 has also been linked also to tumor formation, progression, and metastasis and to the responses to chemotherapeutics. Its expression is elevated in several solid tumors, and HMGB1 serum levels are often associated with worse prognosis [**110, 114**]. On the other hand, HMGB1 plays a role in the immune responses against tumors elicited by conventional therapies. HMGB1 is released from irradiated and doxorubicin-treated tumor cells, and through TLR4, HMGB1 is efficient in activating DCs to cross-present tumor antigens, suggesting a dual role for the molecule [**115, 116**]. The redox state of HMGB1 is important in this context. Reduced HMGB1 binds to RAGE, but not to TLR4, promoting tumor resistance to chemotherapeutic agents such as melphalan, paclitaxel, UV, and oxaliplatin. Oxidized HMGB1, in contrast, apparently increases the cytotoxicity of the agents, with the eventual death of tumor cells [**117**].

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LETTER TO THE EDITOR

ECZEMA AND FOOD ALLERGY IN AN ITALIAN PEDIATRIC COHORT: NO ASSOCIATION WITH TLR-2 AND TLR-4 POLYMORPHISMS

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Recent studies have indicated that Toll-like receptor polymorphisms or their impaired signalling, specifically TLR-2 and TLR-4, were correlated with a higher risk for allergy. The purpose of this study is to evaluate the associations of TRL-2 and TRL-4 single nucleotide polymorphisms (SNP) and atopic traits in a cohort of 159 Italian allergic children (102 affected by eczema and 57 by IgE-mediated food allergy) and 147 healthy controls recruited in Rome, Italy. DNA was isolated from the peripheral blood and TLR-2 R753Q/TLR-4 D299G polymorphisms were determined by TaqMan MGB probes using Real-Time PCR technique. In the control group, the TLR-2 polymorphism R753Q had a prevalence of 2.5% while the frequency of the TLR-4 D299G was 12%. None of the 159 allergic patients showed the R753Q SNP. By contrast, 7/57 patients with food allergy (12%) and 6/102 subjects with eczema (6%) carried the TLR-4 mutation. In our cohort, no evidence of correlation between TLR-2 or TLR-4 polymorphism and eczema and food allergy incidence and/or severity was found. Further studies are needed to clarify the possible role of TLR-2 and TLR-4 polymorphism in allergic disease, in Italian children.

In recent years, many studies have greatly increased our understanding of the immunologic mechanisms involved in the pathogenesis of allergic disease (antigen presentation, effectors and regulatory T cells, histamine, G protein-coupled receptors, IgE, airway remodelling). The hygiene hypothesis, that is still a complex question, suggests that lack of microbial stimulation in early infancy may lead to allergy. Actually, it was observed that infants colonised in the first week(s) of life with *Staphylococcus aureus* have a lower risk of developing food allergies (1). Reduced early microbial exposure has become a leading cause to explain the increasing rate of allergic disease, thus generating intense interest in the interaction between the microbial environment and key microbial recognition pathways, namely Toll-like receptor (TLR) function. TLRs were found on many cells involved in immediate host defence, including neutrophils, natural killer cells, antigen-presenting cells, which play a critical role in adaptive T-cell responses, and also $CD4^+$ $CD25^+$ T cells, which are important in the prevention of hyper-reactive immune responses. It has been suggested that exposure to specific infections drives the maturing immune system in infancy/childhood towards a protective Th1 phenotype rather than the Th2 cell phenotype that is associated with atopy. Therefore,

Key words: eczema, food allergy, polymorphisms, TLR-4, TLR-2

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0394-6320 (2010)

it is possible that a defective expression or genetic alterations of TLR receptor might contribute to an unbalanced level of Th2 and Th1 immune response. Several studies have indicated that polymorphisms or the impaired signalling of TLRs were correlated with a increased risk for allergy in adults and/or children. TLR-4 D299G and TLR-2 R753Q polymorphisms have been associated with asthma and/or atopic eczema (2-4), and their defective signalling led to allergic sensitization to food protein in mice (5-6). Newborns with a high risk of allergy have also been noted to have altered generation of putative regulatory T-cell populations after LPS stimulation, presumably through TLR-4 pathways. Also, TLR-2 mutation has been associated with a higher risk of asthma in European children (2), with atopic dermatitis having severe phenotype (3). By contrast, recent papers demonstrated that polymorphisms in TLR-2 and TLR-4 were not associated with asthma or eczema (7-8).

The purpose of this study is to evaluate the association of TLR-2 R753Q (rs5743708) and TLR-4 D299G (rs4986790) single nucleotide polymorphisms (SNP) and atopic traits in a cohort of 159 Italian allergic children.

MATERIALS AND METHODS

Patients

One hundred and fifty-nine Italian allergic children (102 with eczema and 57 with food allergy) and 147 healthy controls, matched for age and sex, selected consecutively during the period January 2008-May 2009 from S. Pietro Hospital-Fatebenefratelli, Rome, Italy, were enrolled in this study. Eczema was diagnosed following the criteria of Hanifin and Rajka and the clinical severity was assessed by the SCORAD index. One hundred and two (102) subjects, suffered from eczema (64 male; age range months: 3-206, median 54), showed a mild to severe dermatitis phenotype (SCORAD score: median 46 points, range 8-96 points). Fifty-three of 102 children showed IgE mediated eczema (total IgE > 100 UI/ml) (51.9%), 24/102 suffered from asthma as secondary pathology, (23.5%) and 17/102 presented food allergy (16.6%). Regarding food allergy, all 57 children were positive to IgE-mediated provocation test to cow's milk (29 male; age range in months: 6-198, median 58, 57/57 with skin prick tests and prick by prick positive to cow's milk allergens). Thirty-eight of 57 had mainly gastrointestinal symptoms and insufficient growth (66.6%), 19 of 57 developed eczema and /or urticaria.

Allergy tests

The *in vivo* diagnosis was made with biologically standardized extracts of *Dermatophagoides pteronissynus* and *farinae,* mixed grass pollen (*Lolium perenne, Dactylis glomerata, Phleum pratense* and *Festuca pratensis*), cat and dog epithelium, *Parietaria judaica*, tree mix (*Betula alba, Cupressus semprevirens* and *Olea europea*) and the most common food allergens (cow milk, egg, peanut, cod fish, wheat and soybean). Wheals of greater than 3 mm in diameter were considered positive if skin reactivity to a positive and negative control was as expected.

In all patients and controls, total and specific serum IgE for inhalant and food allergens were tested with UniCap IgE Pharmacia (Upjohn, Uppsala, Sweden) according to the manufacture's instructions. The diagnostic gold standard, the provocation test to cow's milk, was performed as previously described (9) in all 57 children affected by food allergy .

Genotyping method

DNA was isolated from the peripheral blood of all subjects by Qiamp Blood Kit (Qiagen, Hilden, Germany). Determination of the TLR-2 R753Q and TLR-4 D299G polymorphism was performed by TaqMan MGB probes (Applied Biosystem) using a real-time PCR allelic discrimination (iCycler iQ™ Real-Time Detection System version 3.021, Biorad). Primers and hybridization probes were designed with Beacon Designer (Biorad) (Table I).

Allelic discrimination PCR reaction was performed in a final volume of 25 µL containing 1 µL of DNA solution, 12.5 µL of TaqMan Universal PCR Master Mix, 0.62 µL of 40X Assay Mix (unlabeled PCR primers and TaqMan MGB probes), and distilled water. In every assay, controls for the wild type and mutations were included. Reaction mixtures were loaded into 96-well plates. The conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation (92°C for 15 s), annealing and extension in one step (60°C for 1 min). The fluorescent yield for the two different dyes was measured and presented in a two-dimensional graph.

Statistics

TLR polymorphism frequencies of allergic patients and controls were compared using the 2 tailed chi-square test. Results were considered statistically significant when the probability of findings was less than 5% ($p < 0.05$).

RESULTS

Considering TLR-2 R753Q, a prevalence of 2.5% was found in healthy controls, while none of the allergic patients displayed the mutation. The

Table I. *Primers and probes for allelic discrimination.*

		Primers for sequencing	
		Forward	**Reverse**
TLR-4 D299G		5'-CCATTGAAGAATTCCGATTAGCATA-3'	5'-CACTCACCAGGGAAAATGAAGAA-3'
		5'-AGTTTGACAAATCTGCTCTAG-3'	5'-CAATAGTCACACTCACCAG-3'
TLR-2 R753Q		5'-TGGAGAACTTCAATCCC-3'	5'-TTTCAGAAAGCACAAAGA-3'
		5'-TGATGAGAACAATGATGCTGCC-3'	5'-TCCTTCCCGCTGAGCCTC-3'
		TaqMam® Probe sequences	
TLR4	**MUT**[b]	5'-CTCGATGGTATTATTG-3'	
	WT[a]	5'-CCTCGATGATATTATT-3'	
TLR2	**MUT**[b]	5'-TGTGTCTTCATAAGTGGGACTTCATTC-3'	
	WT[a]	5'-TGTGTCTTCATAAGCGGGACTTCATTC-3'	

[a] *TaqMan MGB probe labelled with FAM fluorochrome*
[b] *TaqMan MGB probe labelled with VIC fluorochrome*

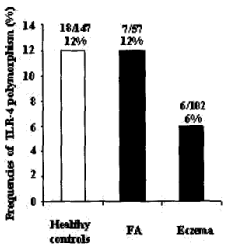

Fig. 1. *Frequencies of TLR-4 polymorphism in 159 allergic children with food allergy (FA) and eczema. Significant differences between allergic patients and the healthy control group were not observed.*

frequency of the TLR-4 polymorphism was 12% in the healthy control group (18 of 147) and 8% (13 of 159) in allergic children, including 7 of the 57 patients with food allergy (12%) and 6 of 102 subjects with eczema (6%) (p=ns for both, Fig. 1). Furthermore, in eczema, no correlation between SNP, IgE level and severe dermatitis phenotype was found. All 6/6 patients with TLR-4 mutations exhibited a phenotype not IgE-associated, characterized by mild eczema (SCORAD score: range, 13-29 points).

DISCUSSION

Our study carried out on an Italian allergic pediatric cohort, suggests that neither TLR-2 nor TLR-4 polymorphisms are associated with atopic diseases. These findings are in agreement with other studies performed in ethnically diverse subjects affected by asthma and eczema, in which no TLR-2 or TLR-4 SNPs were correlated with allergy (7-8). By contrast, other reports demonstrated a possible role of TLR polymorphisms in intensification of susceptibility to infections with various pathogens, defining a subgroup of patients with severe allergy

(3). Since the allergic diseases appear to be strongly influenced by genetic factors, in addition to environmental causes, such results cannot be generalized to diverse allergies in correlation with various populations. Indeed, genetic association studies are strongly influenced by the ethnic and geographic contest in which they are carried out, and consistent differences could be present even in relatively homogeneous areas such as Europe (10). In this contest, association studies in different geographic areas could contribute to better understanding the real role of a given polymorphism in a specific area, and negative studies, such as the present one, may have the role of avoiding over-estimation of positive data.

To our knowledge, the role of TLR-2 and TLR-4 allele variant in human cow's milk allergy is poorly investigated. In food allergy, the mucosal immune system plays an important role and TLR status may influence the development of the intestinal microflora. The multiple signaling through different TLRs in the gastrointestinal mucosa may prevent a dominant role by a single TLR in susceptibility to allergic sensitization to food proteins. Even if the lack of TLR-4 expression is associated with a major susceptibility to food allergy (5), Berin et al. demonstrated that in mice TLR-4 may influence allergic responses positively or negatively according to individual genetic background and the nature of the antigens (6). Our data do not indicate correlation between TLR-2 or TLR-4 polymorphism and cow's milk allergy in our cohort of 57 children.

In eczema, microorganisms and TLRs, that recognize specific molecular signatures on microbes, are likely to play a more important role than in food allergy. Since common features of eczema are cutaneous colonization with *Staphylococcus aureus*, defective host immune defence against microbial pathogens (dysfunctional epithelial barrier, reduced production of antimicrobial peptides and defects in TLR signalling) and high IgE levels, a strict correlation between polymorphism and pathology could be postulated, as reported by other studies (3). By contrast, in our subset of pediatric patients, no association between TLR-2 and TLR-4 polymorphism and eczema prevalence and/or severity emerged. In fact, in our eczema children, TLR-4 polymorphism occurred only in 6% of allergic patients suffering from mild and non-IgE-mediated eczema. One of the possible explanation could be found in the different mutation rate in the control group. In fact, Ahmad-Nejad et al. (3), who demonstrated association between TLR4 polymorphism and eczema, observed a SNP frequency of 11% in 78 patients versus a mutation rate of 2.5% in 39 healthy subjects. The low frequency of control group differs with other studies and our current data, which report frequencies of about 6-14% of TLR-4 D299G non-synonymous polymorphism in Indo-European individuals (11).

In conclusion, our data show for the first time that polymorphisms in TLR2 or TLR4 are not associated with the frequency of food allergy, and no further association between TLRs mutation and atopy were found. We acknowledge that a potential limitation of the present study could be represented by the relatively limited number of subject analyzed. Indeed, the possible association of TLR polymorphism and allergic diseases in Italian children need to be further investigated before conclusions can be drawn.

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HMGB1 and Cord Blood: Its Role as Immuno-Adjuvant Factor in Innate Immunity

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Abstract

In newborn the innate immune system provides essential protection during primary infections before the generation of an appropriate adaptive immune response that is initially not fully operative. Innate immune response is evoked and perpetuated by molecules derived from microorganisms or by the damage/death of host cells. These are collectively known as damage-associated molecular-pattern (DAMP) molecules. High-mobility group box 1 protein (HMGB1) or amphoterin, which previously was considered to be only a nuclear factor, has been recently identified as a DAMP molecule. When it is actively secreted by inflammatory cells or passively released from necrotic cells, HMGB1 mediates the response to infection, injury and inflammation, inducing dendritic cells maturation and T helper-1-cell responses. To characterize the role of HMGB1 in the innate and immature defense mechanisms in newborns, human cord blood (CB) mononuclear cells, in comparison to adult peripheral blood (PB) mononuclear cells, have been analyzed for its expression. By flow cytometry and western blot analysis, we observed that in CB and PB cells: i) HMGB1 is expressed on cell surface membranes of myeloid dendritic cell precursors, mostly, and lymphocytes (gamma/delta and $CD4^+$ T cells) to a lesser extent; ii) different pro-inflammatory stimuli or molecules that mimic infection increased cell surface expression of HMGB1 as well as its secretion into extracellular environment; iii) the treatment with synthetic molecules such as aminobisphosphonates (ABs), identified to be γδ T cell antigens, triggered up-regulation of HMGB1 expression on mononuclear cells, as well γδ T lymphocytes, inducing its secretion. The modulation of its secretion and the HMGB1-mediated migration of monocytes indicated HMGB1 as regulator of immune response in an immature system, like CB, through engagement of γδ T lymphocytes and myeloid dendritic cell precursors, essential components of innate immunity. In addition, the increased HMGB1 expression/secretion triggered by ABs, previously characterized for their immuno-modulating and immune-adjuvant capabilities, indicated that immunomodulation might represent a new therapeutical approach for neonatal and adult pathologies.

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Introduction

The neonatal immune system is generally considered to be immature and less functional compared to adult counterpart. This immaturity is thought to account for the failure of the newborn to mount robust and protective response against several pathogens, resulting in increased mortality [1–4]. The impairment of the newborn immune system may result from the combined effects of a number of factors including: immaturity of its cellular components; lack of previous exposure to antigens; intra-uterine exposure to unique hormonal and cytokine environment which may support Th2 subset development; low proliferation capacity of T lymphocytes and its impaired Th1 cytokine production. Therefore, at the onset of microbial infections, before the generation of an appropriate adaptive (antibody or T cell mediated) immune response, the most important line of defense is innate immunity, where γδ T lymphocytes together with dendritic cells (DCs), macrophages/monocytes and NK cells are the essential components. Innate immunity triggers proinflammatory reactions and is involved in the initial clearance of pathogens.

During the last decade it has been observed that the innate immune response also orchestrates the subsequent adaptive immune response through cytokines and chemokines released by macrophages, DCs and Langerhans cells that are differently activated by the initial innate response. Unlike adaptive immunity, innate immunity is programmed to recognize series of molecular patterns present at the infected lesion: (i) the patterns that are presented by microorganisms [pathogen-associated molecular patterns (PAMPs)], and (ii) the patterns of host intracellular molecules secreted by dying host cells into the extracellular spaces upon microorganism-induced damage [damage-associated molecular patterns (DAMPs)] [5–8]. Consequently, the co-existence of PAMPs and DAMPs signals after invasion by pathogenic microorganisms are closely associated to tissue damage.

The list of DAMPs candidate molecules is getting longer and includes high mobility group box 1 (HMGB1), heat shock proteins, interleukin-1α (IL-1α), defensins, annexins, and S100 [9–13]. HMGB1, or amphoterin, previously has been reported to be only a nuclear factor able to enhance transcription. More recently, HMGB1 has been demonstrated to be a crucial cytokine that

mediates the response to infection, injury and inflammation. HMGB1 is a 30 kD nuclear protein of 215 amino acids. It includes two DNA-binding domains: the A box and the B box, and a negatively charged C-terminal tail. Truncation of HMGB1 indicates that the recombinant A box (1–89) acts as a specific antagonist, whereas the cytokine activity of HMGB1 is determined by the recombinant B box (90–176) [14]. The first 20 amino acids of the recombinant B box represent the minimal peptide maintaining cytokine activity.

HMGB1 recruits inflammatory cells and activates innate immune cells. Further, after release from necrotic cells or its secretion by activated macrophages, it regulates adaptive immunity [13,15–17]. Moreover, HMGB1 supports the maturation and migration of antigen-presenting cells, in particular DCs, to secondary lymphoid organs where these cells play a central role in the activation of naive T cells, in the promotion and induction of Th1 responses, and clonal expansion of antigen-specific T cells, the process at the basis of the adaptive immune response [18,19]. Recently, Kalyan S [20] has reported that peripheral γδ T lymphocytes, previously activated by nonpeptidic antigen isopentylpyrophosphate (IPP), induced the upregulation of CD40 on monocytes and the local release of HMGB1, indicating γδ T cells as immune modulators of stress stimuli and Th1 polarization together with HMGB1. In immune response, γδ T cells represent the first line of defense and are considered to be the border between innate and adaptive immune response. Interestingly, aminobisphosphonates (ABs) which are synthetic compounds commonly used to treat bone disease and hypercalcemia in patients with multiple myeloma, breast or prostate cancer, have been identified also as antigens for γδ T cells, indicating these molecules as immunomodulating factors [21–26].

The expression of HMGB1 and its role in immune response has been demonstrated successfully in adult peripheral blood (PB). Only recently, Buhimashi CS *et al* [27] observed that HMGB1, together with soluble receptor for advanced glycation end-products (sRAGE) and S100, may be important mediators of cellular injury in fetuses and crucial factor in preterm birth induced inflammation. Therefore, due to the importance in understanding immature and innate immune profiles in newborn, we have characterized the expression and modulation of HMGB1 in human cord blood (CB) mononuclear cells.

Results

HMGB1 is expressed on cell-surface of human cord blood cells

Since HMGB1 is present in serum of human cord blood, indicating it as possible mediator of inflammation in fetuses [27], by FACS analysis we first evaluated the intracellular expression of HMGB1 in human CB and PB derived cells in comparison to HeLa cells, known to express HMGB1. Due to the fact that HMGB1 is a nuclear factor, the totality of CB and PB permeabilized cells showed the presence of intracellular HMGB1 expression at a comparable frequency of HeLa cells (Fig. 1A). In addition, we evaluated cell surface expression of HMGB1 in absence of permeabilization, ever since any data was reported on its expression in CB cells. To this end, mononuclear cells isolated from human CB were cultured in complete growth medium and the HMGB1 expressing cells were determined 48 h after isolation. Flow cytometry analysis indicated that 13%±4 (n = 8) CB mononuclear cells expressed HMGB1 on their surface whilst in PB cells HMGB1 was present on 6.5%±1.8 of cells (n = 8) (Fig. 1A). Interestingly, CB cells presented a significantly higher constitutive HMGB1 expression than PB ($p = 0.02$). Fig. 1B shows the overlay of fluorescence histogram plots in one representative

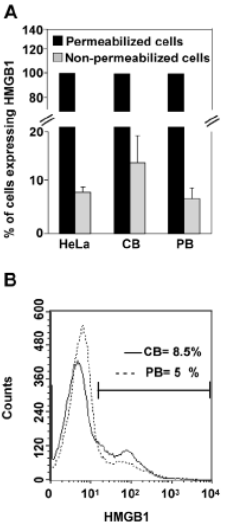

Figure 1. HMGB1 is expressed in human cord blood cells. Mononuclear cells isolated from human cord (CB) and peripheral blood (PB) have been cultured in complete growth medium in absence of external stimuli. (A) The intracellular and surface expression of HMGB1 has been determined 48 h after isolation by flow cytometry analysis in permeabilized and non-permeabilized cells, respectively. HeLa cells represent the reference cell line known to be HMGB1 positive. Values (mean ± SD of eight experiments from different donors) are expressed as percentage of cells labeled with anti-HMGB1 antibody. (B) The fluorescence histogram plot derived from FACS analysis shows the HMGB1 expression profile of CB cells (solid line) and PB cells (dotted line). The percentage of HMGB1 positive cells is indicated in graph. The histogram plot is representative of eight different experiments.
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CB respect to PB, indicating the higher percentage of HMGB1 expressing cells (8.5% in CB versus 5% in PB). In HeLa cells, HMGB1 is expressed by a mean of 7.6%±1 cells.

HMGB1 is expressed mainly on myeloid DC precursors

After 48 h of cell culture in complete growth medium, by multi color flow cytometric analysis we evaluated the cell distribution of HMGB1 expression on different cell subsets of CB and PB. As shown in Fig. 2A, in CB cells about 90% of HMGB1 is expressed on myeloid DC precursors identified in two subsets with $CD14^+CD11c^+$ and $CD14^-D11c^+$ phenotype [28]. Only a small portion of HMGB1 (11%±8) positive cells is represented by $CD3^+$ subset. These results were further corroborated by data achieved in PB, showing a pattern of cell surface HMGB1 expression comparable to CB (Fig. 2A). In Fig. 2B, a representative dual-color FACS plot analysis indicated that the large part of CB $CD11c^+$ cells were positive for HMGB1. On the contrary, only a small fraction of $CD3^+$ expressed HMGB1. Similar data were obtained

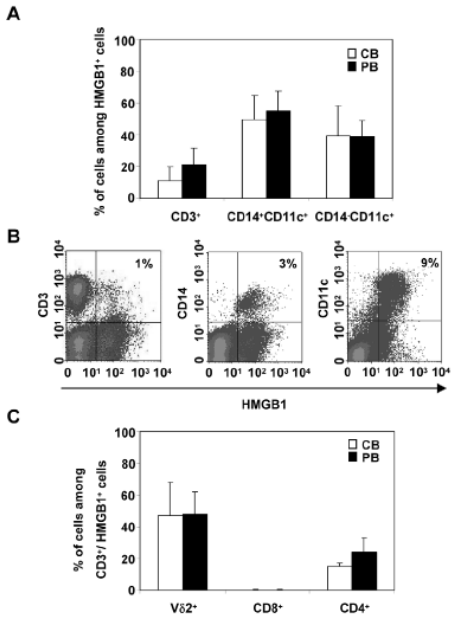

Figure 2. Distribution of HMGB1 cell surface expression: myeloid DC precursors are main cell subset. At 48 h after isolation of mononuclear cells from CB and PB, multi-color flow cytometric analysis has been performed to evaluate surface-expressed HMGB1 and expression of cellular differentiation markers. (A) Two main subsets were identified to be HMGB1 positive in CB and PB: $CD3^+$, lymphocytes; $CD14^+CD11c^+$ and $CD14^-$ $CD11c^+$, myeloid DC precursors. Values (mean ± SD of five experiments from different donors) are expressed as percentage of HMGB1 positive cell subset among the totality of HMGB1 expressing cells. (B) Representative histogram plots derived from two-color FACS analysis show the percentage, indicated on the right of each plot (upper right panel), of $HMGB1^+$ $CD3^+$ (left plot), $HMGB1^+$ $CD14^+$ (middle plot) and $HMGB1^+$ $CD11c^+$ (right plot) in CB cells. Histograms plots are representative of five different experiments. (C) In order to characterize the different T lymphocyte subsets, CB and PB cells have been gated for lymphocytes and multi-color stained with HMGB1, CD3 and gammadelta (Vδ2) or CD8 or CD4 antibodies. The values are the mean ± SD of five experiments from different donors.
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analyzing PB cells (data not shown). In order to identify the various subsets of HMGB1 positive lymphocytes, the $CD3^+$ $HMGB1^+$ cell population has been further characterized by flow cytometry using monoclonal antibodies for CD4, CD8 and TCR gammadelta (Vδ2). As reported in Fig. 2C, cell surface expression of HMGB1 was confined primarily in Vδ2 T cells both in CB and PB. Also $CD4^+$ T-cells resulted positive for HMGB1, whilst $CD8^+$ lymphocytes did not present any expression of HMGB1.

The previous reported data show the expression of HMGB1 on a population of differentiated cells having a high forward scatter, corresponding potentially to myeloid DC precursors. In order to further support them, we analyzed CB and PB cells after 14 days of cell culture because two different cell populations were identified: adherent and non-adherent cells. By FACS analysis, we observed that CB adherent cells expressed a significantly higher levels of HMGB1 than non-adherent (14%±5 versus 7%±3 in non-adherent cells, $P = 0.003$) (Fig. 3A–B). On the contrary, in PB the two cell populations displayed a similar levels of HMGB1 cell surface expression ($P = 0.14$). However, although not statistically significant, the PB adherent cells showed a trend toward higher HMGB1 expression compared to non-adherent cells. The different levels of HMGB1 expression between CB and PB cells observed at 48 h, has been found also after 14 days of culture in the adherent cell subset.

In this context, CB adherent cells shows a shift in the fluorescence curve to the right, indicating a greater expression of HMGB1 in adherent than non-adherent cells (Fig. 3B). On the contrary, in PB cells the level of fluorescence appears only slight different in adherent or non adherent subpopulations (Fig. 3B). Moreover, further characterization by FACS analysis indicated that HMGB1-positive adherent cells were $CD14^+CD11c^+$ and $CD14^-D11c^+$ cells, confirming the results obtained at 48 h (Fig. S1).

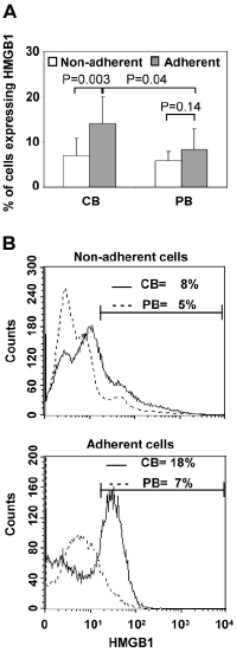

Figure 3. HMGB1 is present mostly in CB adherent cells. Mononuclear cells isolated from human CB and PB have been cultured in complete growth medium. After 14 days, two cell populations have been identified: adherent and non-adherent cells. (A) The cell surface expression of HMGB1 was determined in the two cell populations by flow cytometry analysis. Data are shown as percentage of cells expressing HMGB1 and values are mean ± SD of eight experiments from different donors. Statistical analysis compared non-adherent versus adherent cells or CB versus PB (*P<0.05, **P<0.01 paired Student's t test). (B) The fluorescence histogram plots displays HMGB1 levels in non-adherent and adherent CB (solid line) and PB cells (dotted line) and the percentage of HMGB1 positive cells is indicted in graphs. Data reported is representative of eight different experiments.
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Different stimuli modulate HMGB1 expression and its secretion

In peripheral blood, HMGB1 has been recently demonstrated to be a cytokine secreted by activated immune cells and mediate the response to infection, injury and inflammation. Therefore, we investigated whether various activation signals, such as proinflammatory stimuli (TNF-α or IL-2 or IL-15), or signals that mimic infection (LPS, SEB or PMA), influence the cell surface expression and active secretion of HMGB1 by human cord blood cells. As shown in Fig. 4A, flow cytometry analysis indicated that cell surface expression of HMGB1 was up-regulated by all stimuli. TNF-α increased HMGB1 levels by 2.5-folds in CB and 1.7-folds in PB cells over control at 14 days after treatment. At the same time point, up to approximately 5-folds increase in HMGB1 expression was observed in CB cells treated with IL-15 in contrast to PB cells in which up-regulation reached only 2-folds over control. In addition, IL-2 treatment showed a similar stimulation of HMGB1 expression in CB and PB cells. Similar trend in HMGB1 up-regulation has been observed after 48 h of treatment with stimuli mimicking infection. SEB and PMA determined a similar induction of HMGB1 expression both in cord blood and peripheral blood cells (2-folds), whilst LPS showed a higher increase of protein expression in CB (2-folds) than PB cells (0.8-folds). To determine whether the triggered expression of HMGB1 on cell membrane was associated to its secretion, western blot analysis was performed on the culture medium of CB and PB cells. All different stimuli induced secretion of HMGB1 from CB and PB cells at 48 h and 14 day after treatment (Fig. 4B), while no detectable amounts of HMGB1 has been found in untreated cell medium. Quantitative evaluation of HMGB1 band intensity revealed that IL-2, IL-15 and LPS determined a greater secretion of protein in CB than PB cells (Fig. 4B). On the other hand, TNF-α, SEB and PMA determined the secretion of similar amount of HMGB1 in the two cell types (Fig. 4B). Interestingly, the levels of cell surface expression and secretion of HMGB1 resulted strictly correlated. Moreover, the constitutive and the inducible expression level observed, presented a similar trend toward higher prevalence in CB cells.

To determine whether the modulation of HMGB1 expression in CB cells was associated to different intracellular localization of HMGB1, LPS treated CB cells were co-stained with anti-HMGB1 (stained in green) and membrane-specific PKH26 red fluorescent dye. Confocal immunofluorescence microscopy revealed that in resting CB cells HMGB1 presented heterogeneous labeling pattern, and was localized to the nucleus/cytoplasm, as well as under the apical membrane and faintly on cell surface (Fig. 4C). After forty-eight hours of LPS stimulation, HMGB1 appeared to move from the nucleus/cytoplasm, which is still partly positive, to the periphery of the cells and precisely around its external perimeter, as indicated by the colocalization with membrane-specific PKH26, well evident in the cell with peripheral section (Fig. 4C). This change in surface expressed HMGB1 was completely confirmed by FACS analysis above reported (Fig. 4A).

The constitutive and inducible expression of HMGB1 is regulated via non-classical secretory pathway

Recently, Gardella et al [29], showed that IL-1β and HMGB1 were secreted by monocytes via non-classical secretory pathway and HMGB1 secretion was reduced by Atp Binding Cassette trasporter (ABC-1) inhibitors. To investigate the effect of ABC-1 inhibitor on HMGB1 expression and secretion in CB and PB cells, mononuclear cells were treated with glyburide (100 μM) and/or LPS (0.5 μg/ml). After 20 h, we evaluated cell surface expression and release of HMGB1 by FACS analysis and western blot, respectively. The results showed that glyburide induced a 50% reduction in constitutive expression of HMGB1 on cell surface in CB and PB (P<0.05) (Fig. 5A). In addition, the ABC-1 inhibitor prevented the LPS effect at 20 h after treatment, partially restoring the constitutive cell surface expression of HMGB1 that resulted decreased as consequence of protein release induced by LPS (P>0.05). Western blot analysis indicated that glyburide blocked HMGB1 secretion induced by LPS in CB and PB cells (Fig. 5B). No cytotoxic effect was observed at 24 h after treatment with glyburide by MTT cytotoxicity assay. Altogether, these data

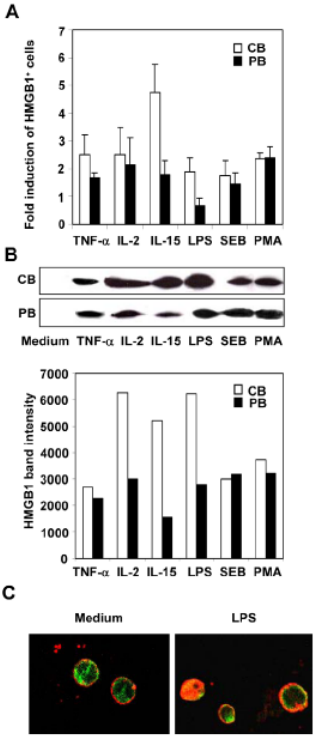

Figure 4. Different stimuli induce cell surface-expressed HMGB-1 and its secretion into extracellular environment. Various activation signals, such as proinflammatory stimuli (TNF-α or IL-2 or IL-15) or that mimic infection (LPS, SEB or PMA) have been used to influence the cell surface expression and active secretion of HMGB1. CB and PB cells have been treated with proinflammatory mediators for 14 days and stimuli that mimic infection for 48 h. (A) Cell surface expression of HMGB1 has been analyzed by FACS analysis, evaluating the percentage of positive cells. In bar graph the values are shown as ratio between treated and untreated cells expressing HMGB1 (fold induction). The values are the mean ± SD of four experiments from different donors. (B) The culture medium of CB or PB cells, which have been analyzed in (A) for surface HMGB1 expression, has been evaluated for its secretion by western blot analysis. The levels of protein shown in (B), quantified by densitometric analysis, has been expressed as arbitrary units. Western blot are representative of three independent experiments. (C) Untreated CB cells or treated with LPS for 48 h were fixed, permeabilized and stained with anti-HMGB1 antibody (green channel) and membrane-specific PKH26 red fluorescent dye. The fluorescence has been analyzed by confocal microscopy (Leica TCS SP5). doi:10.1371/journal.pone.0023766.g004

suggests that the constitutive and inducible expression or secretion of HMGB1 is regulated through alternative, non-classical routes, as also confirmed by enhancing of its expression (2-folds over control) after Brefeldin A treatment (data not shown). The pharmacological modulation of HMGB1 expression was associated to different intracellular localization of HMGB1, as demonstrated by immunofluorescence analysis of CB cells co-stained with anti-HMGB1 (stained in green) and membrane-specific PKH26 red fluorescent dye (Fig. 5C). Confocal immunofluorescence microscopy revealed that in untreated CB cells HMGB1 was localized mainly under and on the apical membrane. Moreover, HGMB1 staining was observed to be concentrated at areas of cell-cell contact. In glyburide treatment HMGB1 became less concentrated and more dispersed resulting in a faint fluorescence, as quantified by FACS analysis. Moreover, the green fluorescence was often punctuate, suggesting cytoplasmic compartmentalization of the protein within vesicles. Twenty hours after stimulation with LPS, HMGB1 displayed a similar pattern of distribution observed after glyburide treatment, confirming the lower cellular expression of HMGB1 in treated than untreated cells observed by FACS analysis. Following the glyburide treatment, the modification of HMGB1 distribution induced by LPS was partially prevented, restoring a distribution of HMGB1 concentrated around the perimeter.

HMGB1 is up-regulated by aminobisphosfonates

Since HMGB1 was expressed on γδ T lymphocytes, we evaluated whether aminonobisphosphonate compounds (ABs), Pamidronate (PAM) and Zoledronate (ZOL), known to induce activation and proliferation of γδ T lymphocytes, were able to trigger HMGB1 expression. After 14 days of cell culture in presence of ABs (1 μg/ml), FACS analysis indicated that cell surface expressed HMGB1 was upregulated by PAM and ZOL, reaching 2.2- and 3.5-folds induction over control in CB and PB cells, respectively, after ZOL treatment (Fig. 6A). Furthermore, both compounds determined an evident expansion of HMGB1 positive γδ T cells which were markedly increased by ZOL treatment (10-folds over control) (Fig. 6B). This data was completely in agreement with evaluation of HMGB1 secretion by western blot analysis performed 14 days after treatment with 1 μg/ml ABs. Both ABs led to a significant induction of HMGB1 secretion into extracellular environment (Fig. 6C). As observed with cell surface expression, ZOL triggered a stronger release of HMGB1 by CB and PB cells than PAM treatment. Even if PAM showed to have a lower effect on upregulation of HMGB1 expression than ZOL, its efficacy on HMGB1 release was significantly increased compared to control. Finally, to demonstrate that ABs-induced release of HMGB1 was not determined by cell death, at 14 day of treatment we evaluated apoptosis/necrosis by flow cytometry analysis after Annexin V/Propidium Iodide staining. No significant change in Annexin V and Propidium Iodide positive cells was observed in ABs treatment in comparison to control.

HMGB1 mediates migration of monocytes

Extracellular HMGB1 acts as immune-stimulatory signal that promotes recruitment of inflammatory cells, as monocytes and dendritic cells, by signaling through RAGE [30]. As we observed HMGB1 release after treatment with different stimuli, pre-conditioned medium generated from the culture of CB or PB cells after 14 days with IL-2 treatment, has been evaluated for its effect on cell migration. After 4 h pre-conditioned medium induced migration of CB or PB $CD14^+$ monocytes through porous membrane. The phenomenon resulted inhibited by using

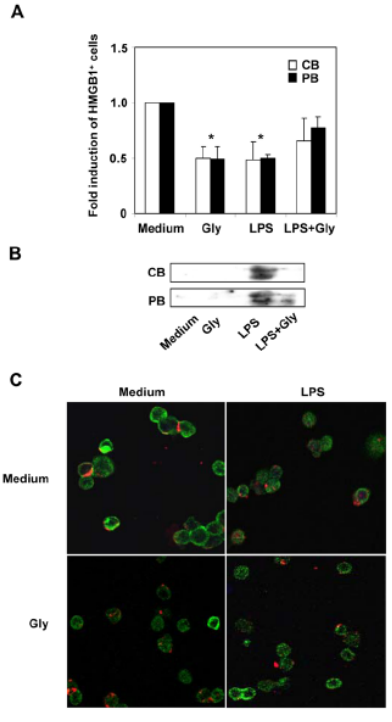

Figure 5. HMGB1 expression is impaired by inhibitor of the Atp binding cassette transporter ABC-1. To characterize the secretion pathway of HMGB1, mononuclear cells were treated with 100 μM glyburide, ABC-1 inhibitor, in presence or absence of LPS (0.5 μg/ml). (A) After 20 h, cell surface-expressed HMGB1 was evaluated by FACS analysis in CB and PB cells. The values are shown as ratio between treated and untreated cells expressing HMGB1 (fold induction). The values are the mean ± SD of three experiments from different donors. Statistical analysis compared treated versus untreated cells (*P<0.05 paired Student's t test). (B) The culture medium of CB or PB cells, which have been analyzed in (A), has been evaluated by western blot analysis to detect its secretion. Western blot is representative of three independent experiments. (C) CB cells were stained with anti-HMGB1 antibody (green channel) and membrane-specific PKH26 red fluorescent dye and analyzed by confocal mycroscopy. HMGB1 expression was evaluated in untreated and glyburide treated CB cells (Top and bottom left panel) or LPS and LPS plus glyburide treated CB cells (Top and bottom right panel) after 20 h of activation.
doi:10.1371/journal.pone.0023766.g005

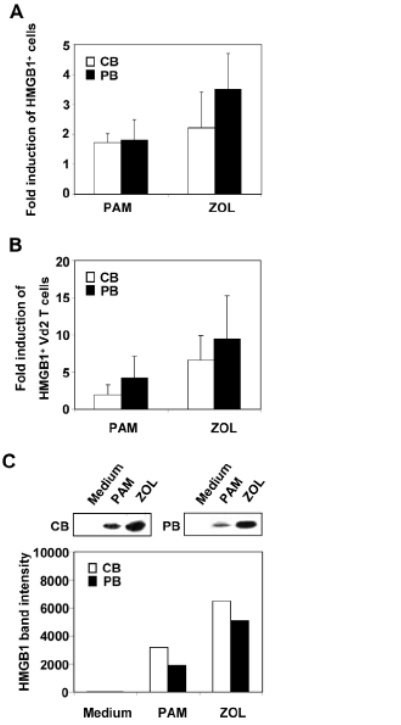

Figure 6. HMGB1 is up-regulated by Pamidronate and Zoledronate in CB and PB cells. Mononuclear cells isolated from CB and PB have been treated with Pamidronate (1 μg/ml) and Zoledronate (1 μg/ml). (A) After 14 days, cell surface expression of HMGB1 has been analyzed by FACS analysis and the values, shown as fold induction, are the product of ratio between treated and untreated cells expressing HMGB1. (B) By multi-color flow cytometric analysis, CB and PB cells have been gated for lymphocytes and stained with HMGB1, CD3 and gammadelta (Vδ2) antibodies. The ratio between treated and untreated $HMGB1^{+}CD3^{+}$ cells is shown as fold induction. The values reported are mean ± SD of four experiments from different donors. (C) The secretion of HMGB1 in cell culture medium was evaluated by western blot analysis. Densitometric analysis of western blot has been expressed as arbitrary units. Western blot is representative of three independent experiments.
doi:10.1371/journal.pone.0023766.g006

N-terminal fragment of HMGB1, Box A (10 μg/ml), as HMGB1 antagonist [31], and anti-RAGE antibody (40 μg/ml) (Fig. 7). Moreover, the percentage of migrated cells resulted lower in presence of anti-RAGE than BoxA, indicating the presence of additional factors that binds RAGE in pre-conditioned medium. The HMGB1-mediated migration of monocytes has been further confirmed by using recombinant HMGB1 protein that induced a similar percentage of migrated cells as observed in pre-conditioned medium in presence of anti-RAGE antibody (data not shown). Finally, the secreted HMGB1 did not induce stem cells chemotaxis, because lacking RAGE expression, which is present

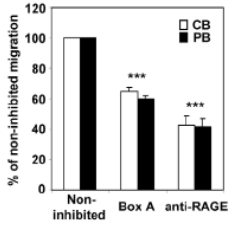

Figure 7. BoxA or anti-Rage antibody partially inhibits monocytes migration. Fresh mononuclear cells isolated from CB and PB have been added to upper well of transwell chamber to measure cell migration induced by pre-conditioned medium generated from the culture of CB or PB cells after 14 days of IL-2 treatment. The migration of monocytes ($CD14^+$ cells) into lower chamber has been evaluated by FACS analysis. Noninhibited migration was defined as 100%. Bars represent mean ± SD of three different experiments. The inhibition of monocytes migration has been obtained by adding BoxA or anti-RAGE antibody in upper well of transwell chamber (***$P<0.001$).
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on monocytes, as assessed by FACS analysis, confirming the inhibitory effect of anti-RAGE antibody on monocytes migration (data not shown).

Discussion

High-mobility group box 1 has been isolated from calf thymus as an abundant nuclear protein over 30 years ago [32]. Recent studies demonstrated that HMGB1 is both actively secreted from activated leukocytes, as a late cytokine mediator [31,33,34] and passively released from necrotic or damaged cells [17]. Therefore, the released HMGB1 acts as trigger of inflammation, attracting inflammatory cells, and tissue repair in autocrine/paracrine fashion.

The importance of HMGB1 as inflammatory mediator examined in adult immune system has been also discovered in fetuses and newborns in which soluble RAGE and HMGB1 are active participants of the tissue injury process [27]. Moreover, in neonates with asphyxia it has been suggested that the elevation of HMGB1 might be associated with abnormal inflammatory responses involving the excessive production of proinflammatory cytokines [35]. The important role of HMGB1 in fetal immune system has been corroborated by data demonstrating that in response to stimuli HMGB1 secreted by human umbilical vein endothelial cells (HUVEC) triggers inflammatory responses through up-regulation of adhesion molecules and release of soluble proinflammatory mediators from endothelial cells [36,37]. Fetal and newborn immune system is characterized to be phenotypically and functionally immature [38]. The innate immune system is the first line of defense against infections in neonates, providing critical protection before the generation of an appropriate adaptive immune response. In consideration of the essential role of neonatal immune responses and the important biological function of HMGB1 as bridge between innate and adaptative immune responses, here we have shown for the first time that HMGB1 is expressed and secreted from mononuclear cells isolated human cord blood. By FACS analysis, we demonstrated that CB cell surface membranes are positive to HMGB1 and its expression is significantly higher in adherent cells than non-adherent cells obtained after long term cell culture. These results show that constitutive HMGB1 expression is confined to a population of differentiated cells, likely myeloid DC precursors in CB and PB. Conversely, lymphocytes, as CD4 or γδ T cells, present a small cell fraction positive for HMGB1. These findings are completely in agreement with the role of HMGB1 in regulating immune response, in which activated monocytes and dendritic cells are the main source of HMGB1 release, promoting their functional maturation in autocrine/paracrine fashion and sustaining the proliferation and polarization of antigen-specific T-cells towards a Th1 phenotype (CD4). γδ T cells represent a small subset of T cells that possess a distinct T cell receptor (TCR) on their surface [39]. These cells exhibit several characteristics that place them, as HMGB1, at the border between the more evolutionarily primitive innate immune system and the adaptive immune system. Thus, our observations give further evidence to the important role of HMGB1 in innate immunity in which macrophages/monocytes, DC and γδ T cells are the main components. Vγ9Vδ2 T cells, which represent the major subset of circulating human γδ T cells, react against a set of non-peptidic, phosphorilated antigens recognized in a TCR-dependent manner [40]. These compounds derive from the mevalonate pathway which is essential for mammalian cells in the sterol synthesis, cell growth and membrane integrity. Other human γδ T cell antigens are synthetic compounds Aminobisphosphonate, such as Pamidronate and Zoledronate [41], known as potent inhibitors of osteoclast-mediated bone resorption used for the treatment of osteoporosis, bone metastasis and cancer [21,42]. It has been shown that bisphosphonates exert a stimulatory effect on adult peripheral blood γδ T cells, *in vitro* and *in vivo*, by inhibiting the mevalonate pathway [43–45]. Recently, we have reported that the treatment with ABs induces proliferative responses in cord blood Vδ2 T cells accompanied by modifications of their naïve phenotype towards a regulatory subset, indicating that they are not inherently unresponsive [46,47]. In this study, we demonstrated that PAM and ZOL trigger cell surfaced expressed HMGB1 in CB and PB cells with an evident increase of HMGB1 positive γδ T cells. Furthermore, ABs treatment leads to remarkable secretion of HMGB1 in extracellular environments. This study provide the first demonstration that ABs treatment modulate the expression of HMGB1 in CB cells, involving Vδ2 T-cells directly or throughout their presentation by APC cells (monocyte lineage) which modulate surface molecules or release cytokines needed for optimal Vδ2 T cell activation, as reported by Miyagawa F et al [48]. Moreover, the enhanced secretion of HMGB1 is not caused by increased apoptosis or necrosis, as demonstrated by FACS analysis. Different stimuli, and not only ABs, are able to modulate HMGB1 expression in CB. In our study, we demonstrated that stimuli that mimic infection (LPS, SEB or PMA) or pro-inflammatory mediators, as TNF-α or IL-2 or IL-15, induce the cell surface expression of HMGB1 and its secretion at 48 h or 14 days after treatment, respectively. Besides, the addition of glyburide, an ABC-1 inhibitor, inhibits LPS-induced secretion, indicating that HMGB1 is released by non-classical secretion pathway, as previously showed by Gardella S. et al [29]. Interestingly, the inhibition of HMGB1 secretion is strictly correlated to its cell surface expression, quantified by FACS analysis, in which ABC-1 inhibitor is able to reduce either its constitutive or inducible expression, demonstrating a role of cell membranes in HMGB1 secretion. As confirmed by confocal microscopy analysis, merged images of LPS treated CB cells verify the almost complete colocalization of HMGB1 and plasma membrane. Moreover, HGMB1 staining is often visualized punctuate, suggesting compartmentalization of the protein within cytoplasmic vesicles and is predominantly localized toward the apical ends, areas of cell-cell contact. Our data are in agreement with data presented by Beer

Stolz D (Pittzburg, PA, USA) and Rouhiainen et al [49], suggesting that HMGB1 is actively secreted from cells by multivescicular endosomes fuse with plasma membrane or that monocytes/macrophages express at cell surface HMGB1, indicating HMGB1 as mediator of cell-to-cell or cell-to-matrix interaction to facilitate their recruitment by binding RAGE at endothelial cells [50].

Results presented herein provide a new insight into the role of HMGB1 in CB innate immune response. We demonstrate that stimuli, as LPS or cytokines or synthetic compounds, can initiate a cascade of events that lead to the activation of immune cells and secretion of mediators, as HMGB1. Once secreted into extracellular milieu, HMGB1 can function as a cytokine to contribute to infectious and inflammatory disorders, as confirmed by our data of its ability on CB monocytes recruitment, mediated by RAGE. Human umbilical vein endothelial cells release HMGB1 and express RAGE. Therefore, HMGB1, which is involved in a paracrine interaction, might play a crucial role in transendothelial migration and consequently in inflammatory immune response in CB.

Whereas a blockade of extracellular HMGB1 might represent a suitable therapeutic target for the treatment of sepsis, the development of the appropriate cell-mediated immunity, which is associated with a Th1 type immune response, is essential for successful immunization. Besides, extracellular HMGB1 has been shown to act as immune adjuvant by enhancing immunogenicity of apoptotic lymphoma cells and eliciting antibody responses to soluble ovalbumin protein [51]. Moreover, a short peptide, named Hp91, identified within the B box domain of HMGB1, induced activation of human and mouse DCs, increasing secretion of pro-inflammatory cytokines and chemokines, including the Th1 cytokine, IL-12 [52]. Therefore, these immunostimulatory properties make HMGB1 an attractive candidate as an adjuvant for vaccine development. The stimulation of neonatal response with vaccines has certain medical advantages and, namely, produce early protection for the vulnerable newborn period. Successful vaccines contain an adjuvant component that activates the innate immune system, thereby eliciting antigen-specific immune responses. Many adjuvants appear to be ligands for toll-like receptors (TLR), as HMGB1, which are promising targets for the development of novel adjuvants to elicit vaccine immunogenicity [53,54]. In this scenario, aminobishoshponates that are able to stimulate innate immunity, as γδ T cells in CB [47], and to induce HMGB1 secretion may be an immuno-modulating tool to approach neonatal pathologies. Moreover, in adult counterpart, considering that ZOL or PAM exert also anti-cancer activity by inducing apoptosis, ABs, as other anticancer agents, may interfere in the complex interaction between tumor and host immune system by the release of inflammatory mediators, such as HMGB1, which mediate cross-presentation of tumor antigens via binding on TLR4 and the promotion of tumor specific cytotoxic T cell responses [55,56]. In conclusion, by modulating the activity of HMGB1 we might provide a potential therapeutic target in adult and neonatal pathologies.

Materials and Methods

Ethics statement

Human umbilical cord blood and buffy coats from peripheral blood donations samples were collected after obtaining informed consent. The consent was written and approved from all participants. All blood samples were only collected from donors that had consented scientific use of blood products. Ethics Committee approval for this study is not required according to institutional guidelines. In particular, approval from the Ethics Committee of our institution was not necessary because blood samples were obtained in compliance with Italian legislation and donors gave informed written consent to donation for research purpose in case of sample with cell content numerically unsuited for clinical use, and therefore are considered as residual sample or waste material. Informed written consent has been requested to the donors or to the authorized parents.

Preparation of cells and culture conditions

Cord blood (CB) was obtained from healthy mothers according to institutional guidelines. Anonymous buffy coats from peripheral blood (PB) donations were collected from healthy blood bank donors. CB samples were obtained from spontaneous partum and normal full-term pregnancies by venipuncture of umbilical vein immediately after delivery. Samples were collected at S. Pietro Fatebenefratelli hospital in Rome. Cord blood was diluted (1:1) with phosphate-buffered saline, PBS (Dulbecco's Phosphate Buffer Saline), and further diluted (1:1) with a solution of 4% dextran in PBS (Sigma Aldrich, St Louis, USA). After sedimentation of erythrocytes, CB and PB samples was layered over Ficoll-Hypaque (Sigma-Aldrich, St. Louis, USA) density gradient and centrifugated at 1,800 rpm for 20 minutes. Freshly isolated mononuclear cells of CB and PB were cultured at 3×10^6/ml in RPMI 1640 (Sigma Aldrich) supplemented with 10% FBS (Sigma Aldrich), 2 mM L-glutamine (Sigma Aldrich), 10 UI/ml penicillin-streptomycin (Sigma Aldrich).

HeLa cells (ATCC CCL-2) human cervical carcinoma, were cultures in DMEM (Sigma Aldrich) supplemented with 10% FBS (Sigma Aldrich), 2 mM L-glutamine (Sigma Aldrich), 10 UI/ml penicillin-streptomycin (Sigma Aldrich).

Cell treatment

Fresh CB and PB cells were treated with the following stimuli: IL-2 (20 ng/ml) (Roche), TNF-α (20 ng/ml) (eBioscience, Inc), IL-12 (20 ng/ml) (eBioscience, Inc) or aminobisphosphonates, Pamidronate and Zoledronate (Zometa, Novartis Pharmaceutical) (1 μg/ml) for 14 days; Lipopolysaccharide (LPS) (500 ng/ml) (Sigma Aldrich), Staphylococcus aureus Enterotoxin B (SEB) (1 μg/ml) (Sigma Aldrich) or Phorbol 12-myristate 13-acetate (PMA) (30 ng/ml) (Sigma Aldrich) for 48 h. Untreated mononuclear cells have been cultured in complete growth medium for 48 h and 14 days. For secreted HMGB1 detection, culture supernatants were microcentrifuged at 1200×g for 5 minutes and frozen at −70°C until analysis.

Flow Cytometric Analysis of Surface-Expressed HMGB-1 and Expression of cellular differentiation markers

Surface expressed HMGB1 has been analyzed by flow cytometry (FACSCalibur Flow Cytometry System, BD BioSciences Pharmingen) using the anti rabbit-HMGB1 antibody (Sigma Aldrich) and secondary FITC-conjugated antibody (BD Biosciences) or Alexafluor 647–conjugated antibody (Molecular Probes, Invitrogen). The surface expression of cell differentiation markers have been evaluated with the following fluorescently-conjugated antibodies: PE anti-human CD14 (M5E2), PE-Cy5 anti-human CD11c (B-ly6), PE-Cy7 anti-human CD16 (3G8), PE anti-human Vδ2 (B6), PE anti-human CD8 (SK1), FITC anti-human CD4 (RPA-T4) (BD Biosciences, PharMingen) and APC anti-human CD3 (UCHT1) (eBioscience, Inc). The fluorescence labelling has been performed by incubating cells at 4°C (protected from light) in PBS with 4% bovine serum albumin for 30 minutes with antibodies. Cells were subsequently washed, resuspended in 500 μl PBS and acquired on flow cytometer (FACSCalibur, BD Biosciences). 100000 events have been collected and analyzed by Cell Quest program (BD Biosciences).

For Intracellular staining of HMGB1, CB and PB cells were fixed and permeabilzed with Cytofix/Cytoperm solution (BD Cytofix/Cytoperm Kit, BD BioSciences Pharmingen) prior to the addition of anti-HMGB1 antibody.

Western Blot Analysis of Secreted HMGB1

CB and PB cell culture supernatants or complete culture medium (100 µl) were boiled in reducing Laemmli sample buffer, resolved on 12% SDS/PAGE under reducing conditions and electrotransferred onto PVDF filters (Hybond-P, Amersham Pharmacia Biotech, Milan, Italy), which were stained with Ponceau S (Sigma) and de-stained prior to blocking with 5% non-fat dry milk in PBS containing 0.05% Tween (Sigma) for 1 h. Filters were stained with polyclonal rabbit anti-HMGB1 antibody overnight at 4°C followed by anti-rabbit IgG horse-radish-peroxidase-(HRP-) conjugated secondary antibody for 1 hour at room temperature. HMGB1 detection was performed using Super Signal substrate (Pierce, Rockford, IL) according to the manufacturer's instructions. Quantitative evaluation of HMGB1 protein was determined by densitometric analysis.

Inhibition of HMGB1 expression and secretion by ABC-1 inhibitor glyburide

In order to characterized the secretion pathway of HMGB1, CB and PB mononuclear cells (3×10^6 cells/ml) have been activated with LPS (500 ng/mL) in presence of 100 µM glyburide (Sigma), a potent inhibitor of the secretion via Atp binding cassette transporter (ABC-1). After 20 hours HMGB1 has been evaluated as cell surface expression and its secretion in the culture medium by FACS and western blot analysis, respectively, as indicated before. The potential cell toxic effects of glyburide and its solvent (dymethylsulfoxide, DMSO) were determined by MTT.

Confocal fluorescence microscopy

CB and PB mononuclear cells (3×10^6 cells/ml) have been deposited on glass slides by centrifugation at 400 rpm for 5 minutes using a cytospin system (Thermo Shandon, Pittsburgh, PA). They were air dried, fixed in cold 4% paraformaldehyde for 15 minutes. Cells were washed in PBS and permeabilized in 0.2% Triton X-100 in PBS for 10 min at 4°C and then blocked with 1% bovine serum albumin in PBS for 40 min. Immunofluorescence staining of cells was performed using rabbit anti-HMGB1 antibody (1:300) and anti-rabbit-FITC conjugated secondary antibody (1:200). Antibodies dilutions in PBS containing 0.1%BSA were added to cells and incubated for 1 h at room temperature. After washing in PBS, general cell membrane labeling PKH26-GL red fluorescent dye (1:250 dilution; Sigma) was added for 10 min at room temperature. Cells were washed three times in PBS, mounted using ProLong Gold antifade reagent (Molecular Probes, Invitrogen) and then analyzed using confocal microscope (Leica TCS SP5). Software: LAS AF version 1.6.3 (Leica Microsystem).

MTT assay

Cytotoxicity has been quantified by measurement of the reduction of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltrazolium bromide, a tetrazole) (Promega) to produce a dark blue formazan product. MTT has been added to each well 20 h after the beginning of the insult. After 3 h incubation, the Solubilization/Stop Solution has been added to the culture wells to solubilize the formazan product, and the absorbance at 570 nm recorded using a 96-well plate reader (Bio-Rad® Laboratories)

Detection of apoptosis

CB and PB mononuclear cells (1×10^6 cells/mL) were washed with ice-cold PBS and the cell pellets were resuspended in ice-cold binding buffer. Five µL Annexin V FITC solution and 5 µL propidium iodide (Bender MedSystems, Austria) were added to 490 µL of the prepared cell suspension and incubated at 4°C for 10 min in the dark. Aliquots were directly aspirated into a FACSCalibur flow cytometer and apoptosis analysed by CellQuest program.

Monocytes chemotaxis assay

Fresh mononuclear cells isolated from CB or PB were placed to upper compartment of the Transwell chamber (3-µm pore size) and pre–conditioned medium, generated from the culture of CB or PB cells after 14 days of treatment and centrifugation at 1200×g, was added in lower well. After 4 h, migrated cells were characterized by FACS analysis. BoxA (HMGBiotech, Milan, Italy) or anti-RAGE antibody (Millipore, USA) were used to inhibit HMGB1 mediated cell migration.

Statistical Analysis

Student's *t*-test (one-tail) has been used to assess the significance of differences in HMGB1 expression. Differences were considered significant if the probability of the null hypothesis was less than five percent (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Supporting Information

Figure S1 Adherent cells are myeloid DC precursors expressing HMGB1. At 14 days after isolation from CB and PB, adherent mononuclear cells were characterized by multi-color flow cytometric analysis of surface-expressed HMGB1 and expression of cellular differentiation markers. $CD14^+CD11c^+$ and $CD14^-CD11c^+$ phenotypes are adherent cells expressing HMGB1. Values (mean ± SD of five experiments from different donors) are expressed as percentage of HMGB1 positive cell subset among the totality of HMGB1 expressing cells.
(TIF)

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Author Contributions

Conceived and designed the experiments: AC IG VC GM. Performed the experiments: AC IG ZAP. Analyzed the data: AC IG ZAP GM. Contributed reagents/materials/analysis tools: AC IG ZAP EA GM. Wrote the paper: AC VC ZAP GM.

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CONCLUSIONS

The present studies demonstrate important roles of TLRs and HMGB1 in neonatal and child's immune system.

The identification and functional characterization of TLRs in Drosophila and mammals have brought our understanding of the innate immune system to a new level. The role of the TLRs in host defense is fundamental, it is likely that their function affects most aspects of the mammalian immune system. Loss-of-function mutations in TLRs result in immunodeficiencies, whereas gain-of-function mutations might predispose an individual to inflammatory or autoimmune disorders. The importance of the TLRs in the control of adaptive immune responses also makes them crucial targets for immune intervention. Therefore, complete understanding of the mechanisms of innate immunity will be helpful for the future development of innovative therapies for manipulation of infectious diseases, cancer and allergies. Owing to the important role of innate immunity in neonatal health and disease, the intense biopharmaceutical development of molecules that are derived from or that modulate the innate immune system, including antimicrobial proteins, peptides and TLR agonists, could have clinical relevance to neonatal medicine. Therefore, TLR agonists might represent tools to enhance the defense against microorganisms [**118, 119**] or to shift innate immune responses of neonatal APCs away from the production of TH2-cell-polarizing cytokines, thereby potentially reducing allergy [**120, 121**].

Actually, several studies have indicated that polymorphisms of TLR-4 and TLR-2 are associated with allergy, asthma or atopic eczema [**71-73**] and its defective signaling led to allergic sensitization to food protein in mice [**74, 75**]. High risk newborns for allergy have also been noted to have altered generation of putative regulatory T-cell populations after LPS stimulation, presumably through TLR-4 pathways [**72**]. Also TLR-2 mutation has been associated with a higher risk for asthma in European children [**73**] and with atopic dermatitis having severe phenotype [**76, 77**]. Conversely and In agreement with some recent papers [**122, 123**], we have observed that specific polymorphisms in TLR-2 and TLR-4 are not associated with eczema and food allergy in Italian allergic children, indicating that correlation between disease and TLR polymorphism might influence allergic responses positively or negatively as a function of the individual genetic background and the nature of the associated antigens.

Recently, Ferhani et al. [**124**] reported that levels of HMGB1, that is a DAMP molecule and TLR-4 ligand, were also elevated in the fluid from

bronchoalveolar lavage of patients with chronic obstructive pulmonary disease. Straub et al. [**125**] reported that HMGB1 inhibitors significantly diminished the ovalbumin-induced increase in response to methacholine in a mouse asthmatic model sensitized and challenged with ovalbumin. Moreover, it was suggested that measurement of HMGB1 and soluble RAGE (sRAGE) might be novel biomarkers in asthma with severe airflow limitation [**126**]. These reports suggest HMGB1 as an important mediator of asthma. Asthma and atopic allergies are considered inflammatory disorders, therefore a blockade of extracellular HMGB1 might represent a suitable therapeutic intervention for the treatment of these pathologies.

The importance of HMGB1 as inflammatory mediator examined firstly in adult immune system has been also discovered in fetuses and newborns in which sRAGE and HMGB1 are active participants of the tissue injury process [**127**]. The important role of HMGB1 in fetal immune system has been corroborated by data demonstrating that in response to stimuli HMGB1 secreted by human umbilical vein endothelial cells (HUVEC) triggers inflammatory responses through up-regulation of adhesion molecules and release of soluble proinflammatory mediators from endothelial cells [**15, 104**]. The expression of HMGB1 and its role in immune response has been demonstrated successfully in adult PB and only recently, Buhimashi CS et al [**128**] proposed that HMGB1, together with soluble receptor for advanced sRAGE and S100, are important mediators of cellular injury in fetuses and crucial factor in preterm birth induced inflammation. Here we have shown for the first time that HMGB1 is expressed and secreted from mononuclear cells isolated from human cord blood. We demonstrated that constitutive HMGB1 expression is confined to a population of differentiated cells, likely myeloid DC precursors in CB and PB. Conversely, lymphocytes, as CD4 or γδ T cells, present a small cell fraction positive for HMGB1. These findings are completely in agreement with the role of HMGB1 in regulating innate immune response, in which activated macrophages/monocytes, DC and γδ T cells are the main components. Moreover, we demonstrated that stimuli that mimic infection or pro-inflammatory mediators induce the cell surface expression of HMGB1 and its secretion via non-classical secretion pathway. HGMB1 is compartmentalized within cytoplasmic vesicles during active release and is predominantly localized toward the apical ends, areas of cell-cell contact, as mediator of cell-to-cell or cell-to-matrix interaction to facilitate cell recruitment by binding RAGE at endothelial cells.

Interesting results were obtained by cell treatment with ABs, that we have recently demonstrated to be inducers of proliferative responses in cord blood Vδ2 T-cells [**36, 37**]. Pamidronate and Zoledronate are able to trigger cell

surface expression of HMGB1 in CB and PB cells with an evident increase of HMGB1 positive γδ T cells. Furthermore, ABs treatment leads to remarkable secretion of HMGB1 in extracellular environments. This study provide the first demonstration that ABs treatment modulate the expression of HMGB1 in CB cells, involving Vδ2 T cells directly or throught their presentation by APC cells (monocyte lineage) which modulate surface molecules or release cytokines needed for optimal Vδ2 T-cell activation, as reported by Miyagawa F et al [**129**].

Results presented herein provide a new insight into the role of HMGB1 in CB innate immune response. We demonstrate that stimuli, such as LPS or cytokines or synthetic compounds, can initiate a cascade of events that lead to the activation of immune cells and secretion of mediators, as HMGB1. Once secreted into extracellular milieu, HMGB1 can function as a cytokine/chemokine recruiting CB monocytes via RAGE. Human umbilical vein endothelial cells release HMGB1 and express RAGE. Therefore, HMGB1, which is involved in a paracrine interaction, might play a crucial role in transendothelial migration and consequently in inflammatory immune response in CB.

To date a number of HMGB blocking therapies have been tested in several experimental models of different diseases. Recent structure-based drug discovery efforts have been aimed at increasing the number of small molecule- and biologics-based prototype therapeutics targeting HMGB1. Small molecule drugs that may provide therapeutic benefit through HMGB1-directed mechanisms involve HMGB1 inhibitory ligands, Toll-like receptor antagonists, RAGE antagonists, alpha7 nicotinic acetylcholine receptor agonists, G2A antagonists, serine protease inhibitors, and alpha-dicarbonyl-based soft electrophiles. Using some of these agents, pharmacological modulation of HMGB1-associated cutaneous pathology has been achieved with an acceptable toxicity profile, and preclinical proof-of-concept experimentation has demonstrated feasibility of developing HMGB1-modulators into novel systemic and topical therapeutics that target inflammatory dysregulation. The identification of HMGB1 inhibitor, glycyrrhizin, is therefore of significant experimental and clinical interest. Glycyrrhizin binds to high-mobility group box 1 protein and inhibits its cytokine activities. Mollica et al [**130**] show that, a natural anti-inflammatory and antiviral triterpene in clinical use, inhibits HMGB1 chemoattractant and mitogenic activities, and has a weak inhibitory effect on its intranuclear DNA-binding function. Recently, a new derivative of glycyrrhizin is used as HMGB1 scavenger in clinical therapy of rhinitis via nasal spray.

Whereas a blockade of extracellular HMGB1 might represent a suitable therapeutic target for the treatment of inflammation, the development of the appropriate cell-mediated immunity, associated with a Th1 type immune response, is essential for successful immunization practice. Extracellular HMGB1 has been shown to act as immune adjuvant by enhancing immunogenicity of apoptotic lymphoma cells and eliciting antibody responses to soluble ovalbumin protein [**89**]. Moreover, a short peptide, named Hp91, identified within the B box domain of HMGB1, induced activation of human and mouse DCs, increasing secretion of pro-inflammatory cytokines and chemokines, including the Th1 cytokine, IL-12 [**131**]. Therefore, HMGB1 might be considered a probable candidate as adjuvant for vaccine. In this scenario, aminobishoshponates that are able to stimulate innate immunity, as γδ T-cells in CB [**37**], and to induce HMGB1 secretion may be an immuno-modulating tool to approach neonatal pathologies. Moreover, in adult counterpart, considering that ZOL or PAM exert also anti-cancer activity by inducing apoptosis, ABs might interfere in the complex interaction between tumor and host immune system by the release of inflammatory mediators, such as HMGB1, which mediate cross-presentation of tumor antigens via binding on TLR4 and the promotion of tumor specific cytotoxic T-cell responses [**132**]. The ability of immuno-adjuvant to induce antigen-specific cell mediated, Th1 immune response, may make it suitable as an adjuvant in cancer immunotherapies as well as in vaccines against infectious diseases caused by intracellular bacteria or viruses.

Thus HMGB1 appear to be a double-edged sword. While being vital for tissue repair or immuno-adjuvant because it exerts stimulatory effect on immune system, it also plays a role in the pathogenesis of many inflammatory and autoimmune diseases that feature aberrant TLR activation. HMGB1–TLR–RAGE constitutes a tripod. Blockade or downregulation of HMGB1, or control of the inflammatory tripod, represent a promising therapeutic approach for the treatment of adult and neonatal pathologies.

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