kinases and phosphatases

MDPI

Communication

Regulation of Extracellular HMGB-1 Alarmin Levels by CIGB-300 Anticancer Peptide In Vitro and In Vivo

Daylen Aguilar-Noriega [1,†], Ying Yi [2,†], Jamilet Miranda [3], Yanelda García [4], Dania M. Vázquez [1], Yaqin Lan [2], Ricardo Bringas [3], Wen Li [2,*], Yasser Perera [1] and Silvio E. Perea [2,*]

[1] Biomedical Research Area, Center for Genetic Engineering and Biotechnology (CIGB), Havana 10600, Cuba; daylen.aguilar@cigb.edu.cu (D.A.-N.); dania.vazquez@cigb.edu.cu (D.M.V.); yasser.perera@cigb.edu.cu (Y.P.)
[2] Research Department, China Cuba Biotechnology Joint Innovation Center (CCBJIC), Yongzhou 425000, China; ivy@ccbjic.com (Y.Y.); ava@ccbjic.com (Y.L.)
[3] Bioinformatic Department, Center for Genetic Engineering and Biotechnology (CIGB), Havana 10600, Cuba; jamilet.miranda@cigb.edu.cu (J.M.); ricardo.bringas@cigb.edu.cu (R.B.)
[4] Clinical Research Department, Center for Genetic Engineering and Biotechnology (CIGB), Havana 10600, Cuba; yaneldag@cim.sld.cu
* Correspondence: liwen@ccbjic.com (W.L.); silvio@ccbjic.com (S.E.P.)
† These authors contributed equally to this work.

Abstract

HMGB-1 is an alarmin representative of DAMP playing a central role in immunogenic cell death (ICD), a necessary condition in the dialog established between dying tumor cells and the immune system during some anticancer therapies. Therefore, early screening for ICD inducers represents a major priority in drug development today. In this work, we investigated the effect elicited by the clinical-grade CIGB-300 peptide, which impairs Protein Kinase CK2-mediated phosphorylation and other CK2 signaling connected kinases. Here, HMGB-1 extracellular release was investigated in an 18-cell line panel from blood malignancies, uterine-cervical cancer and NSCLC treated with CIGB-300 at equipotent doses (IC_{50}) over 24 h. Interestingly, CIGB-300 treatment upregulated the HMGB-1 protein levels at the culture supernatant in most of the cell lines ($p = 0.01$) and fold-change increases ≥ 2 were associated with intrinsic cell line sensitivity towards CIGB-300's cytotoxic effect. However, the HMGB-1 release by CIGB-300 was context-specific with clear induction on blood and uterine-cervical cancer cells and a diffused response pattern in NSCLC. Importantly, CIGB-300 treatment of blood cancer patients enrolled in a Phase I study induced plasma HMGB-1 alarmin in 4 out of 7 subject who received the entire treatment plan. Altogether, our data reveal for the first time that CIGB-300 treatment is able to induce extracellular HMGB-1 release in vitro and in vivo which could be indicative of ICD induction in some kinds of tumors; furthermore, the induction of extracellular HMGB-1 alarmin as a putative CIGB-300 response biomarker merits further investigation.

Keywords: immunogenic cell death; protein kinase CK2; HMGB-1; CIGB-300

Check for updates

Academic Editor: Marco Falasca

Received: 22 June 2026
Revised: 11 August 2026
Accepted: 14 August 2026
Published: 18 August 2026

Copyright: © 2026 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

1. Introduction

High mobility group box 1 (HMGB-1) is a highly conserved nuclear protein that plays an essential role in chromatic condensation and transcriptional regulation [1]. In addition to its nuclear function, HMGB-1 has a well-characterized function in the extracellular environment where it acts as a damage-associated molecular pattern (DAMP) to mediate inflammation and immune responses [2]. HMGB-1 release can occur passively, as a consequence of cell necrosis, or actively through regulated secretion in different cell types,

including activated immune cells, necrotic cells, and certain types of tumor cells. This process is tightly controlled by post-translational modifications, including phosphorylation, acetylation, and oxidation, which determine its translocation from nucleus to cytoplasm and its subsequent release [3]. On the other hand, high levels of HMGB-1 alarmin have been reported in patients with solid tumors and blood cancer [4,5].

Casein kinase 2 (CK2) is a constitutively active serine/threonine kinase that is frequently overexpressed in multiple types of cancer [6]. CK2 regulates a wide range of key cellular processes, including proliferation, apoptosis, stress response, and intracellular signaling, making it an important therapeutic target [7]. Recent evidence indicates that CK2 is also involved in the regulation of nuclear proteins and in the modulation of inflammatory pathways [8,9], suggesting a connection with the mechanisms that control the release of HMGB-1. In this regard, different studies have demonstrated the effect of CK2-mediated phosphorylation of HMGB-1 on modulating its stability, DNA binding activity [10,11] and the subsequent shuttles between nucleus and cytoplasm [12]. Despite these advances, the effect of CK2 inhibition on HMGB-1 release in tumor cells has not been fully elucidated.

Several strategies for CK2 enzyme inhibition have been evaluated in pre-clinical studies. Of these, only two have emerged as promising tools for both research and therapeutic applications, the synthetic-peptide CIGB-300 and the ATP-competitive inhibitor CX-4945 (Silmitasertib). CIGB-300, currently under clinical evaluation, is a chimeric peptide containing a cell-penetrating moiety that impairs CK2-mediated phosphorylation through targeting the phosphoacceptor domain in the substrates [13] or direct binding to the enzyme [14]. On the other hand, CX-4945 is a selective ATP-competitive CK2 inhibitor orally bioavailable, indicated by the FDA as an "orphan drug" for cholangiocarcinoma in 2017 [15]. Both inhibitors have demonstrated anti-proliferative and antineoplastic effect in solid tumor and hematological malignancies [16–19].

Despite these advances, the specific role of CK2 inhibition in regulating HMGB-1 alarmin release and subsequent ICD induction remain insufficiently characterized. In particular, the effect that the clinical grade CIGB-300 and CX-4945 inhibitors might elicit and consequent inflammatory response is unclear. Thus, this study aims to investigate "a priori" the effect of pharmacological inhibition of CK2 using the CIGB-300 peptide on the induction of HMGB-1 release in leukemia, non-small cell lung cancer (NSCLC) and cervical cancer cell lines as a previous step to subsequent investigation of ICD induction.

2. Results

CIGB-300 Treatment Induces HMGB-1 Release

To investigate the putative effect of CIGB-300 on the extracellular HMGB-1 alarmin release, a panel containing 18 tumor cell lines from different origins were assayed in the presence of two CK2 inhibitors, the CIGB-300 peptide and CX-4945, after 24 h of treatment. For these assays, the cells lines were treated with equipotent doses (IC_{50}) eliciting cell death by both compounds (Table 1).

Interestingly, CIGB-300 but not CX-4945 significantly induced the extracellular levels of the HMGB-1 alarmin in most of the tumor cell line panels ($p = 0.01$ versus $p = 0.34$) (Figure 1a).

The segregated analysis showed that on blood cancer cell lines ($n = 5$), HMGB-1 was detected in the culture supernatant of all cell lines after treating with CIGB-300 IC_{50} and levels were at least twice as high as those found in untreated condition ($p = 0.043$) (Figure 1b). Otherwise, a non-significant effect was observed for CX-4945 with just a mild increase in extracellular HMGB-1 on HL-60 cells. Thus, equipotent dose levels of both CK2 inhibitors, which elicits 50% of cell death, differ in their ability to increase the HMGB-1 extracellular levels on these leukemia cell lines. To investigate whether induction

of extracellular HMGB-1 levels was correlated with putative regulation of the alarmin intracellular levels by CIGB-300, total cell extracts from HL60 and OCI-AML3 cell lines treated with CIGB-300 or CX-4945 were prepared and analyzed by Western blot. Of note, neither CIGB-300 nor CX-4945 equipotent doses (IC_{50}) seemed to affect the HMGB-1 intracellular levels at these experimental conditions (Figure 2).

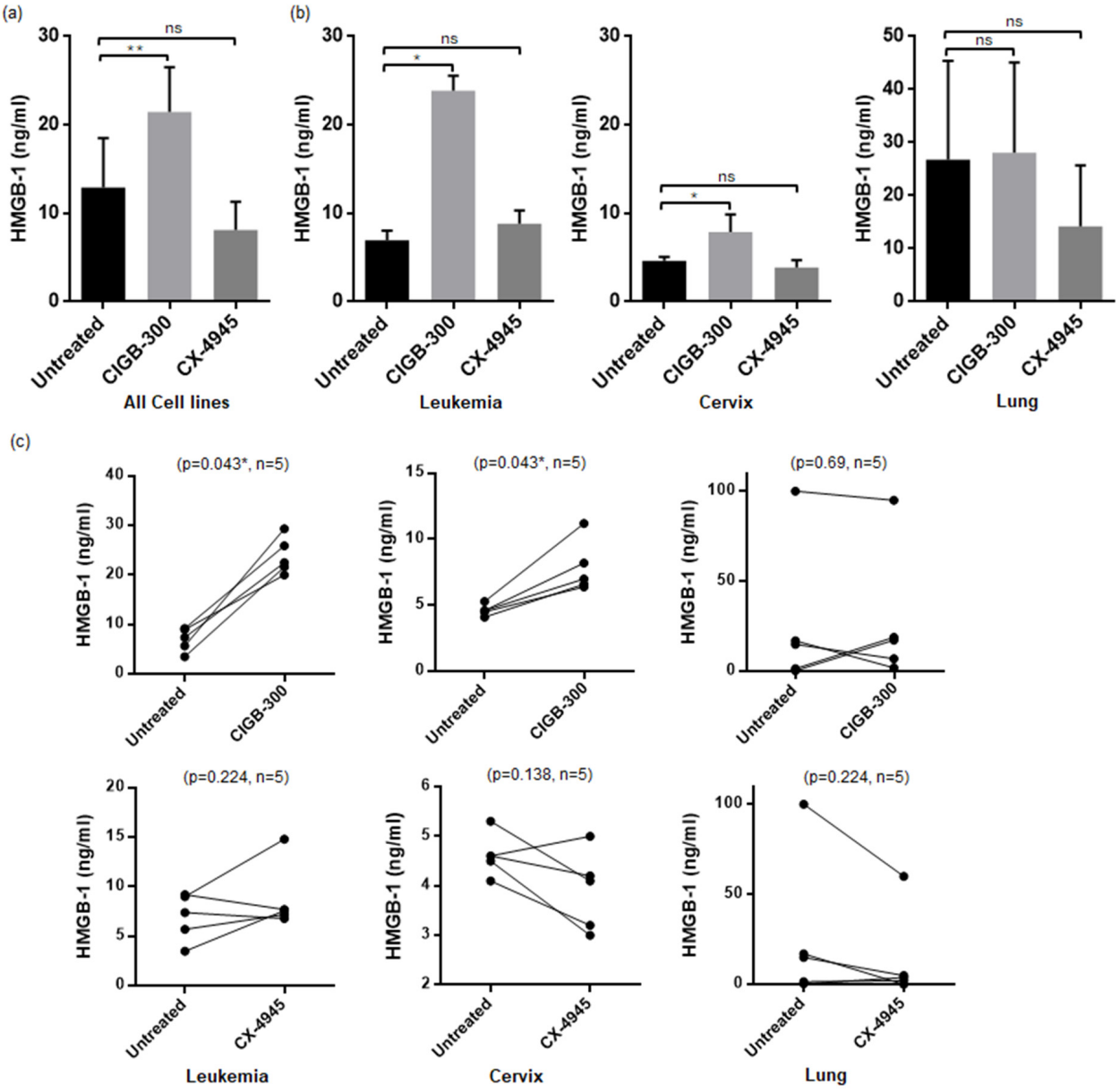


Figure 1. HMGB-1 release levels induced by CIGB-300 or CX-4945 in different tumor cell lines for 24 h. Cell lines were treated with equipotent doses (IC_{50}) of CIGB-300 and CX-4945 shown in Table 1 for 24 h. (**a**) HMGB-1 release levels of all cell lines involved in this study (n = 18). (**b**) HMGB-1 release levels of cell lines grouped by their origins: blood (Leukemia), uterine cervical cancer and NSCLC. (**c**) Individual effect according to the tumor cell line origin. Extracellular HMGB-1 levels were measured by ELISA as indicated in Material and Methods. Column graphs represent mean + SEM (standard error of mean). Statistical tests contrasting treated versus untreated paired samples were performed (Wilcoxon Matched Pairs Test). Significance is represented as * $p \leq 0.05$; ** p 0.01; ns, not significant.

Table 1. Concentrations of CIGB-300 and CX-4945 that inhibit 50% of cell proliferation in the cell lines used in the research.

Cell Lines	IC_{50} CIGB-300 (μM)	IC_{50} CX-4945 (μM)
HL60	37	4.9
OCI-AML3	26	6.3
THP1	19	5
K562	33	4.9
L1210	40	13.4
NCI-H125	70	5.3
NCI-H460	30	5
A549	90	11.5
NCI-H522	108	9.3
SK-MES-1	140	6.9
C4-1	200	12
CaSki	81	10
HeLa	126	9.4
SiHa	134	6.8
Hep2C	70	7.5
MC38	270	5
MRC5	195	4.7
PBMC	40	11

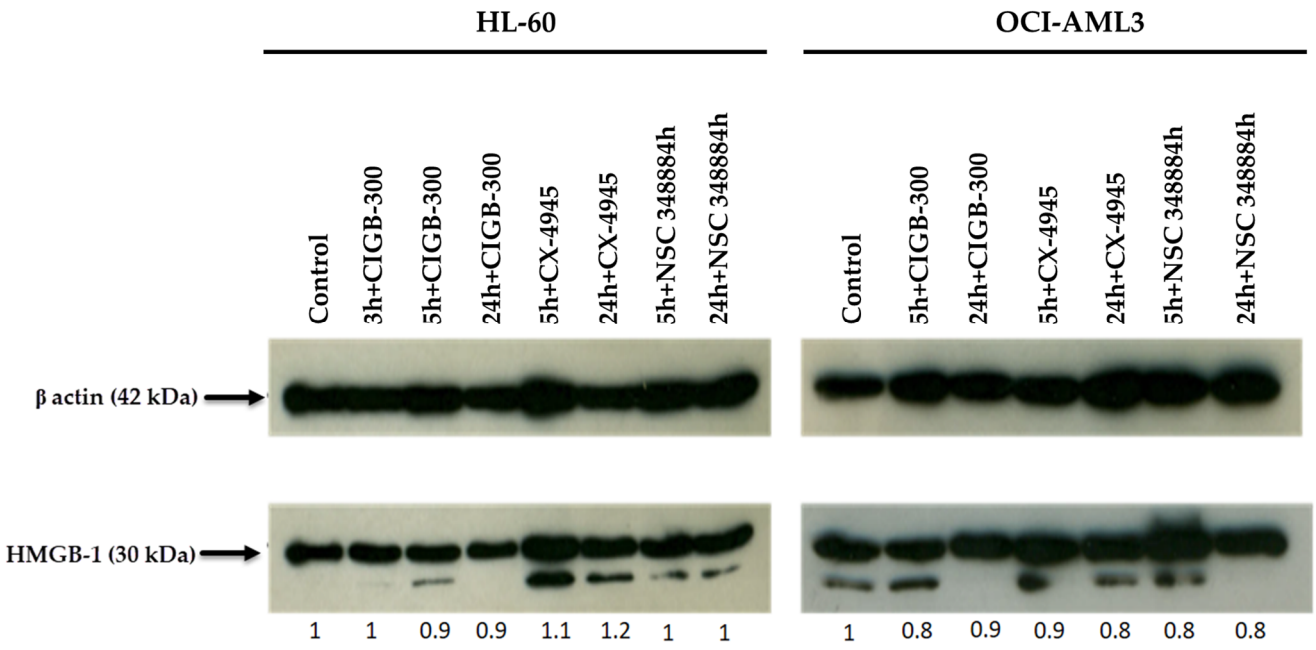


Figure 2. CIGB-300 treatment does not modify the HMGB-1 intracellular levels. HL60 and OCI-AML cell lines were treated with CIGB-300 and CX-4945 IC_{50} at indicated times. The cell extracted was prepared and analyzed by Western blot as indicated in Materials and Methods. The figure shows the result of one representative experiment out of two performed.

We next investigated whether the induction of HMGB-1 release is also observed by treating epithelial tumor cells with CIGB-300. To do that, cell lines from uterine-cervix and lung cancer were treated with IC_{50} equipotent doses of the CIGB-300 peptide and CX-4945 for 24 h.

Similarly to leukemia, extracellular HMGB-1 levels were detected in the supernatant of all cervical cancer cell lines treated with CIGB-300 ($p = 0.034$). The highest levels were detected in the supernatant of the HeLa and Hep2C cell lines (Figure 1b). Conversely, the

addition of CX-4945 to the culture of cervical cancer cells did not induce HMGB-1 release; instead a very modest inhibition in CaSki, SiHa and HeLa cells was observed.

Contrary to what was observed for leukemia and uterine-cervical cancer cell lines, a heterogenous response profile was observed for NSCLC cells treated with CIGB-300 (Figure 1b). CIGB-300 treatment increased the extracellular levels of HMGB-1 by more than 50% on NCI-H460 and NCI-H125 cell lines (Figure 1b) and no effect was elicited by CX-4945. Interestingly, neither CIGB-300 nor CX-4945 modified the extracellular HMGB-1 levels on A549 cells whose baseline levels were notably high. Otherwise, both CIGB-300 and CX-4945 inhibited HMGB-1 release in H522 and SK-MES-1 cell lines. This heterogenous response profile contributed to the non-significant increase in the HMGB-1 levels after CIGB-300 in NSCLC cell lines compared to blood cancer and uterine cervical cancer.

To look for putative associations between the HMGB-1 release induced by CIGB-300 and other parameters like intrinsic cell sensitivity towards CIGB-300, HMGB-1 baseline levels and cancer type, Spearman's rank correlation analysis was used in the 18-cell line panel. A moderated association was observed for HMGB-1 release fold-change after 24 h of CIGB-300 treatment with a negative correlation between the HMGB-1 increase and IC_{50} values ($p = 0.043$, corr = -0.482) (Table 2). Otherwise, association between the CIGB-300 intrinsic cell sensitivity and the baseline or drug-induced HMGB-1 levels was not observed (Table 2).

Table 2. Non-parametric correlation between the CIGB-300 response variable (IC_{50}-CIGB-300) and the expression of HMGB-1 at 24 h.

			HMGB-1_untreated Condition (24 h)	HMGB-1_treated Condition (24 h)	FC_HMGB-1_treated vs. Untreated (24 h)
Spearman's rho	IC_{50}_300	Correlation Coefficient	0.110	−0.197	−0.482 *
		Sig. (2-tailed)	0.664	0.432	0.043
		N	18	18	18

Notes: * $p \leq 0.05$.

Once we had evidence that CIGB-300 treatment induced extracellular levels of the HMGB-1 alarmin in vitro, we subsequently investigated whether in vivo treatment is able to elicit a similar effect. For that purpose, we analyzed the plasma HMGB-1 levels from blood cancer patients enrolled in a Phase I clinical trial who received an in-patient CIGB-300 dose-escalation treatment consisting of 1.6, 3.2, 5.4, 8.6 and 12.8 mg/kg of weight. Interestingly, after completing the CIGB-300 regime for 6 weeks, 4 of out 7 patients who received the entire treatment plan experienced at least a 2-fold increase in plasma HMGB-1 levels compared to baseline (Table 3). Analysis was not performed in three patients who did not complete all the CIGB-300 dose-levels because of disease progression or abandonment of the study.

Table 3. Plasma HMGB-1 levels in blood cancer patients treated with CIGB-300.

Patient Code	Diagnosis	Baseline (ng/mL)	After CIGB-300 Treatment	Fold-Change
AMC-01	Elderly AML	4.04	13.05	3.23
AMC-04	Elderly AML	*	*	NA
GAL-01	Elderly AML	3.60	2.24	0.62
HHA-02	MDS	0.10	7.12	71.2

Table 3. *Cont.*

Patient Code	Diagnosis	Baseline (ng/mL)	After CIGB-300 Treatment	Fold-Change
HHA-04	MDS	2.44	14.4	5.9
HHA-03	ALL	2.44	1.71	0.70
HHA-05	ALL	7.02	20.23	2.88
AMC-02	Refractory AML	11.0	*	NA
AMC-03	Refractory AML	4.10	*	NA
HHA-01	Relapsed AML	1.87	2.38	1.27

Notes: AML (Acute Myeoloide Leukemia), MDS (Myelodisplasic Syndrome), ALL (Acute Lymphocytic Leukemia. * Patients who did not complete all the CIGB-300 dose cycles. NA (Non-Applicable).

3. Discussion

Some anticancer strategies today focus on the transformation of cold- into hot-tumors by creating an immune-inflamed phenotype favored by drug-induced ICD, which can be optimal for efficient immunotherapy [20]. Likewise, early screening for anticancer drugs, which induce immunogenic instead of tolerogenic cell death, is a major priority during drug development today. The exploration of extracellular ICD key biomarkers on anticancer drug-treated cells and in plasma from patients treated with anticancer therapy is the first step towards confirmation of ICD induction by anticancer drugs. Particularly, HMGB-1 is a key DAMP released during early stages of ICD, crucial for stimulating an effective anticancer immune response [21,22].

The induction of apoptosis and cell cycle arrest by inhibiting the CK2-mediated phosphorylation have been experimentally validated by using different kinds of CK2 inhibitors. However, the knowledge of the type of cell death produced by the different CK2 inhibitors and their possible connection with the immune system remains to be elucidated. In this work we explored the effect of CIGB-300 anticancer peptide, which impinges the CK2-mediated phosphorylation via different mechanisms on HMGB-1 alarmin release by tumor cells. Of note, treatment with CIGB-300 IC_{50} significantly induced the HMGB-1 alarmin extracellular levels in most of the cell lines from the panel ($p = 0.01$), with significant effect on blood and uterine-cervical cancer cell lines and heterogenous response on NSCLC. Interestingly, blood cancer cells which showed the greatest sensitivity towards CIGB-300 cytotoxic effect (lowest IC_{50} values) exhibited at least 2-fold induction of extracellular alarmin release regardless the leukemia type. Likewise, integration of results in the entire 18-cell line panel indicated a moderated negative correlation between intrinsic high cell sensitivity to the cytotoxic effect of CIGB-300 (lowest IC_{50} values) and extracellular HMGB-1 release fold-change after 24 h of CIGB-300 treatment. These results merit further confirmation in a broader cell panel.

Although HMGB-1 is a substrate of CK2 [10,12], it was not found among the CK2 substrates inhibited when the CIGB-300-regulated phosphoproteome was interrogated in AML cells such as HL-60 and OCI-AML3 [14]. In addition, the HMGB-1 intracellular levels were not visibly changed by CIGB-300 treatment in both cell lines according to Western blot analysis. This evidence suggests that the phosphorylation of HMGB-1 by CK2 does not play a causal role during its extracellular release induced by CIGB-300; instead, such release seems rather to be a consequence of the cellular reprogramming mechanisms driving cell death where the inhibition of other CK2 substrates by CIGB-300 could play a central role. Furthermore, in the above-mentioned phosphoproteomic analysis, a significant number of phosphosites attributed to other CK2-sequentialy activated kinases such as to glycogen synthase kinase-3beta (GSK3B), members of mitogen-activated protein kinases (MAPKs)

and cyclin-dependent kinases (CDKs) families, also appeared to be modulated in HL-60 and OCI-AML3 by CIGB-300 [14], which could also be connected with the molecular and cellular events driving cell death and HMGB-1 release. Nevertheless, whether CIGB-300 directly affects other HMGB-1 passive release mediators like PARP-1, RIPK3, Cathepsin, DNase, and Caspase [23] merits further investigation.

Additionally, previous studies using microarray, qPCR, and ELISA confirmed that treatment with the CIGB-300 peptide increases TNFα at both transcriptional and protein synthesis levels in HL-60 and OCI-AML3 cells. TNFα was detected in the culture supernatant of both cell lines, with the highest levels found in HL-60 [24]. TNFα appears to be involved in HMGB-1 release into the extracellular environment. In assays performed on monocytes and macrophages stimulated with LPS, the simultaneous release of TNFα and HMGB-1 was detected. In that work, the researchers demonstrated that direct TNFα suppression by genetic TNFα knockout or TNFα-neutralizing antibodies partially inhibits LPS-induced HMGB-1 secretion in macrophages, suggesting that HMGB-1 secretion could be partially mediated through a TNFα-dependent mechanism [25]. In line with this, some genes involved in the NF-κB pathway were also upregulated by CIGB-300 in the same study [24]. Therefore, we cannot rule out the putative role of NF-κB/TNF-α in the HMGB-1 alarmin release induced by CIGB-300 at least in both acute myeloid leukemia cells.

Considering that blood cancer cells responded efficiently to the effect of CIGB-300, we verified whether the in vivo treatment was capable of inducing levels of the plasma HMGB-1 alarmin in blood cancer patients who had participated in a Phase 1 clinical study. Despite the limited population size, four out of seven patients who completed the five-planned CIGB-300 intravenous infusion cycles showed an increase in HMGB-1 alarmin levels $\geq$ 2-fold. Although very preliminary, these data indicate that CIGB-300 treatment is able to induce extracellular HMGB-1 alarmin levels both in blood cancer cell lines and patients. However, this effect seems to be context-specific as some patients remained refractory, something that also happened in the analysis of the cell panel in vitro.

Contrary to what was observed for blood and uterine-cervical cancer, NSCLC cell lines showed a diffuse response pattern towards the CIGB-300 effect where induction of extracellular HMGB-1 alarmin levels was not clearly documented. In fact, only two cell lines (NCI-H125 and NCI-H460), the most sensitive ones to the cytotoxic effect of CIGB-300, exhibited increases in HMGB-1 levels at the supernatant by treating with equipotent CIGB-300 doses; the A549 cell line showed no CIGB-300 effect on the HMGB-1 levels and instead, along with the two other cell lines (H552 and SK-MES-1), experienced a reduction in the extracellular HMGB-1. Interestingly, for the A549 cell line which exhibited high extracellular HMGB-1 baseline levels, CIGB-300 failed to modify the alarmin release. Similar to AML cells, in the phosphoproteomic analysis on NCI-H125 cells, the CK2 phosphosite on the HMGB-1 protein was not found to be inhibited by CIGB-300 treatment among the CK2 substrate pool [26], which rules out a putative instrumental role for the CK2-mediated phosphorylation on HMGB-1 in the subsequent release to the extracellular compartment.

In addition to the mechanisms involved in the active secretion of HMGB-1, it is known that it can be secreted passively by the induction of cell death and increased permeability of the cytoplasmic membrane [3]. Accumulated evidence has shown the induction of cell death by both CK2 inhibitors evaluated in this work [27,28], which would support the passive release of HMGB-1 after treatment with these molecules. However, here the treatment with CX-4945 at IC_{50} dose only induced modest release of HMGB-1 in the HL60 and OCI-AML3 leukemia cell lines. In the other cell lines studied, CX-4945 did not promote the release of HMGB-1 or, as observed in some cases, inhibited its release, since extracellular HMGB-1 levels on CX-4945-treated cells were lower than those belong to baseline. This functional discrepancy suggests that CIGB-300 but not CX-4945 could regulate some of the

molecular events linked to passive extracellular release of HMGB-1. In line with this notion, the HSP90AA1 protein which regulates the translocation of HMGB1 from the nucleus to the cytoplasm and its secretion through direct interaction [23] is differentially regulated by CIGB-300 and CX-4945 as observed in phosphoproteomic assays on OCI-AML3 y HL-60 [26,29]. On the other hand, previous studies reported that high CX-4945 doses (100 μM) induced other ICD biomarkers like ATP release as monotherapy, and calreticulin exposure only in combination with Temozolomide on glioblastoma cells [30]. We found no previous reports investigating the effect of CX-4945 on HMGB-1 release on tumor cells. Whether CX-4945 induces HMGB-1 release at dose levels over IC_{50} and/or longer treatment time requires further clarification.

Despite its dual role in cancer, extracellular HMGB-1 constitutes a paradigmatic biomarker of ICD which plays a central role in the dialog established between dying cancer cells and the immune system in cancer therapy due to its well-characterized function as a DAMP [22]. The results obtained in this work show for the first time that CIGB-300 IC_{50} doses induce HMGB-1 alarmin release in a context-specific way and treatment of blood cancer patients with CIGB-300 can also increase the plasma HMGB-1 alarmin levels. However, further studies are required to determine the redox status of HMGB-1 secreted after treatment with the CIGB-300 peptide, since its chemoattractant [31], cytokine [32], or tolerogenic [33] activity depends largely on the oxidation state of the molecule. Also, our results do not rule out that in addition to the inhibition of signaling the CK2, other kinases inhibited by CIGB-300 as part of the in vivo signal amplification contribute to the process of cellular reprogramming entailing cell death and HMGB-1 release. A comprehensive experimental setting will be required for the final assessment of ICD induction by CIGB-300.

4. Materials and Methods

4.1. Cell Lines and Cultures

The human (HL-60 (ATCC CCL-240), OCI-AML3 (Cytion 305432), THP-1 (ATCC TIB-202), K562 (ATCC CCL243)) and mouse (L1210 (ATCC CCL-219)) leukemia cell lines and peripheral blood mononuclear cells (PBMC) were cultured in RPMI 1640 Medium (Cat. No. 13018-031; Gibco, Waltham, MA, USA) supplemented with 10% (*v/v*) fetal bovine serum (FBS) (Cat. No. FBS-11A; Capricorn Scientific GmbH, Ebsdorfergrund, HE, Germany) and 50 μg/mL gentamicin (Cat. No. G-3632; Sigma, St. Louis, MO, USA). The human epithelial lung (NCI-H460 (ATCC HTB-177), NCI-H125 (ATCC CRL-5801), A549 (ATCC CCL-185), NCI-H522 (ATCC CRL-5810), SK-MES-1 (ATCC HTB-58)), colon (MC38; Kerafast, ENH204-FP) and cervical (C4-1 (ATCC CRL-1594), CaSki (ATCC CRL-1550), SiHa (ATCC HTB-35), HeLa (ATCC CCL-2), Hep2C (ATCC CCL-23)) cancer cell lines were cultured in Dulbecco's Modified Eagle medium (DMEM) (Cat. No. 12800-082; Gibco, Waltham, MA, USA) supplemented with 10% (*v/v*) FBS and 50 μg/mL gentamicin. All cell lines, except MC38 cell line, were originally obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). MC38 cells were obtained from Kerafast (Boston, MA, USA).

4.2. Cell Viability Assay

Cytotoxic effect was determined using AlamarBlue assay (Cat. No. DAL1025, Invitrogen, Life Technologies, Eugene, OR, USA). Cells were seeded in flat-bottom 96-well plates (leukemia cells at 2×10^5 cells/mL, 100 uL/well and epithelial cells at 6×10^4 cells/mL, 100 uL/well), and 24 h later, serial dilutions 1:2 ranging from 200 to 12.5 μM of CIGB-300 and serial dilutions 1:2 ranging from 50 to 3.12 μM of CX-4945 were added. After 48 h of incubation, AlamarBlue was added at 10% (*v/v*) and cells were further incubated for 4 h. Fluorescence was measured in a CLARIOstar microplate reader (BMG LABTECH,

Ortenberg, Germany) and half-inhibitory concentration (IC_{50}) values were estimated using CalcuSyn software (v2.1) (Biosoft, Cambridge, UK).

4.3. Quantification of HMGB-1 Release

The human epithelial cells were planted in 24-well plates with 0.5 mL full medium, which was changed 24 h later and treatment was added onto the cells. In the case of leukemia cell lines, these were seeded at 0.5×10^6 cells/mL in 12-well plates with 1 mL full medium. The cells were treated with CIGB-300 peptide and CX-4945 for 24 h at a dose equivalent to the IC_{50}, determined as indicated above. Supernatants were collected 24 h later, dying cells removed by centrifugation and supernatants isolated and frozen immediately. Quantification of HMGB-1 in the cell culture supernatant or plasma from blood cancer patients who had been enrolled in a CIGB-300 Phase I clinical study was assessed using commercial ELISA kits obtained from IBL International GmbH (Cat. No. 30164033, Hamburg, Germany) and Shanghai Mlbio Technology Co., LTD (Cat. No. ml-31296, Shanghai, China) according to the manufacturer's instructions.

4.4. Intracellular Levels of HMGB-1 on Tumor Cells

HL-60 and OCI-AML 3 cell lines were incubated with CIGB-300 and CX-4945 IC_{50} levels during 3, 5 and 24 h or during 5 and 24 h respectively. Cells were lysed in RIPA buffer containing protease/phosphatase inhibitor (Cat. No. 89901; Thermo Fisher Scientific, Waltham, MA, USA), and equal amounts of protein were resolved in 12.5% SDS-PAGE. Next, proteins were transferred to a nitrocellulose membrane and immunoblotted with the following antibodies according to instructions from the manufacturer: HMGB-1 (Cat. No. ab 18256; abcam, Cambridge, UK) and β-actin (Cat. No. A2228; Sigma, St. Louis, MO, USA). Detection was performed with peroxidase conjugated anti-mouse (Cat. No. A0168; Sigma, St. Louis, MO, USA) or anti-rabbit IgG (Cat. No. A6154; Sigma, St. Louis, MO, USA), and signal was developed using SuperSignal West Pico Chemiluminescent Substrate (Cat. No. 34080; Thermo Fisher Scientific, Waltham, MA, USA).

4.5. Soluble HMGB-1 Levels in Plasma from the EHPMA Clinical Study

An open, adaptative, and multicenter Phase I CIGB-300 in-patient dose-escalation clinical trial had been previously performed in ten patients with blood malignancies like Acute Myeloid Leukemia (AML), Acute Lymphocytic Leukemia (ALL) or Myelodysplastic Syndrome (MDS). CIGB-300 regimen consisted of five consecutive 3 day cycles with escalated dose at 1.6. 3.2, 5.3, 8.0, 12.8 mg/kg and a 4 day-resting period between cycles. Plasma samples were taken after every single cycle; however, the HMGB-1 levels were investigated at baseline and after concluding the entire treatment plan at week 6. The EHPMA clinical study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Center for State Control of Medicines, Equipment and Medical Devices (CECMED) (protocol code IG/CIGB-300I/LAR/1101 and date of approval: 5 November 2014).

4.6. Statistical Analysis

The HMGB-1 soluble values of untreated and drug-treated cell lines were compared using the Wilcoxon matched pairs test. Spearman's rank correlation was used to estimate the association between CIGB-300 response and HMGB-1 expression levels. Statistical analyses were performed with Statistica version 8.0 and SPSS version 13.0. The level of significance used was $\alpha = 0.05$. Prism GraphPad Version 6.0 Software was used for data visualization.

Author Contributions: D.A.-N. (Investigation, methodology, written original draft), Y.Y. (investigation, methodology), J.M. and R.B. (data analysis), Y.G. (methodology in EHPMA clinical study), D.M.V. and Y.L. (methodology and data analysis), W.L. and Y.P. (conceptualization and supervision), S.E.P. (conceptualization, supervision, writing—review, project administration and funding acquisition). All authors have read and agreed to the published version of the manuscript.

Funding: The present research was supported by a grant from the Science and Technology Innovation Program of Hunan Province, China, (grant: 2024RC4027). Also, this work was supported in part by a CITMA-Cuba grant: PN385LH007-013.

Institutional Review Board Statement: The EHPMA clinical study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Center for State Control of Medicines, Equipment and Medical Devices (CECMED) (protocol code IG/CIGB-300I/LAR/1101 and date of approval: 5 November 2014).

Informed Consent Statement: Oral and Written informed consent was obtained from patients to be enrolled at the EHPMA clinical study. Patient's names were blinded by internal codes of the study as a rule of the Good Clinical Practices.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Acknowledgments: Authors want to acknowledge Evelin Caballero for her technical support in this work.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

HMGB-1	High Mobility Group Box 1
DAMP	Damage Associated Molecular Patterns
ICD	Immunogenic cell death
CK2	Casein Kinase 2
NSCLC	Non-Small Cell Lung Cancer
IC_{50}	half-inhibitory concentrations
AML	Acute Myeoloide Leukemia
MDS	Myelodisplasic Syndrome
ALL	Acute Lymphocytic Leukemia
GSK3B	glycogen synthase kinase-3beta
MAPKs	mitogen-activated protein kinases
CDKs	cyclin-dependent kinases

References

1. Lv, G.; Yang, M.; Gai, K.; Jia, Q.; Wang, Z.; Wang, B.; Li, X. Multiple functions of HMGB-1 in cancer. *Front. Oncol.* **2024**, *14*, 1384109. [CrossRef] [PubMed]
2. Yuan, J.Y.; Guo, L.; Ma, J.T.; Zhang, H.J.; Xiao, M.X.; Li, N.; Gong, H.; Yan, M. HMGB-1 as an extracellular pro-inflammatory cytokine: Implications for drug-induced organic damage. *Cell Biol. Toxicol.* **2024**, *40*, 55. [CrossRef] [PubMed]
3. Chen, R.; Kang, R.; Tang, D. The mechanism of HMGB-1 secretion and release. *Exp. Mol. Med.* **2022**, *54*, 91–102. [CrossRef] [PubMed]
4. Yang, H.; Zhao, X.; Li, X.; Wang, X.; Pu, Y.; Wei, D.; Li, Z. A pan-cancer analysis of the oncogenic function of HMGB-1 in human tumors. *Biochem. Biophys. Rep.* **2024**, *40*, 101851. [CrossRef] [PubMed]
5. Rapoport, B.L.; Steel, H.C.; Theron, A.J.; Heyman, L.; Smit, T.; Ramdas, Y.; Anderson, R. High Mobility Group Box 1 in Human Cancer. *Cells* **2020**, *9*, 1664. [CrossRef] [PubMed]
6. Trembley, J.H.; Kren, B.T.; Afzal, M.; Scaria, G.A.; Klein, M.A.; Ahmed, K. Protein kinase CK2—Diverse roles in cancer cell biology and therapeutic promise. *Mol. Cell. Biochem.* **2022**, *478*, 899–926. [CrossRef] [PubMed]

7. Nuñez de Villavicencio-Diaz, T.; Rabalski, A.J.; Litchfield, D.W. Protein kinase CK2: Intricate relationships within regulatory cellular networks. *Pharmaceuticals* **2017**, *10*, 27. [CrossRef] [PubMed]
8. Singh, N.N.; Ramji, D.P. Protein kinase CK2, an important regulator of the inflammatory response? *J. Mol. Med.* **2008**, *86*, 887–897. [CrossRef] [PubMed]
9. Liu, D.; Marie, J.C.; Pelletier, A.L.; Song, Z.; Ben-Khemis, M.; Boudiaf, K.; Pintard, C.; Leger, T.; Terrier, S.; Chevreux, G.; et al. Protein kinase CK2 acts as a molecular brake to control NADPH oxidase 1 activation and colon inflammation. *Cell. Mol. Gastroenterol. Hepatol.* **2022**, *13*, 1073–1093. [CrossRef] [PubMed]
10. Stemmer, C.; Leeming, D.J.; Franssen, L.; Grimm, R.; Grasser, K.D. Phosphorylation of Maize and Arabidopsis HMGB Proteins by Protein Kinase CK2α. *Biochemistry* **2003**, *42*, 3503–3508. [CrossRef] [PubMed]
11. Taira, J.; Higashimoto, Y. Evaluation of in vitro properties of predicted kinases that phosphorylate serine residues within nuclear localization signal 1 of high mobility group box 1. *J. Pept. Sci.* **2014**, *20*, 613–617. [CrossRef] [PubMed]
12. Silva, I.C.d.A.d.; Carneiro, V.C.; Maciel, R.d.M.; Costa, R.F.M.d.; Furtado, D.R.; Oliveira, F.M.B.d.; Silva-Neto, M.A.C.d.; Rumjanek, F.D.; Fantappié, M.R. CK2 phosphorylation of Schistosoma mansoni HMGB-1 protein regulates its cellular traffic and secretion but not its DNA transactions. *PLoS ONE* **2011**, *6*, e23572. [CrossRef] [PubMed]
13. Perea, S.E.; Baladrón, I.; Valenzuela, C.; Perera, Y. CIGB-300: A peptide-based drug that impairs the protein kinase CK2-mediated phosphorylation. *Semin. Oncol.* **2018**, *45*, 58–67. [CrossRef] [PubMed]
14. Rosales, M.; Pérez, G.V.; Ramón, A.C.; Cruz, Y.; Rodríguez-Ulloa, A.; Besada, V.; Ramos, Y.; Vázquez-Blomquist, D.; Caballero, E.; Aguilar, D.; et al. Targeting of protein kinase CK2 in acute myeloid leukemia cells using the clinical-grade synthetic-peptide CIGB-300. *Biomedicines* **2021**, *9*, 766. [CrossRef] [PubMed]
15. Siddiqui-Jain, A.; Drygin, D.; Streiner, N.; Chua, P.; Pierre, F.; O'Brien, S.E.; Bliesath, J.; Omori, M.; Huser, N.; Ho, C.; et al. CX-4945, an orally bioavailable selective inhibitor of protein kinase CK2, inhibits prosurvival and angiogenic signaling and exhibits antitumor efficacy. *Cancer Res.* **2010**, *70*, 10288–10298. [CrossRef] [PubMed]
16. Perea, S.E.; Reyes, O.; Baladrón, I.; Perera, Y.; Farina, H.; Gil, J.; Rodriguez, A.; Bacardi, D.; Marcelo, J.L.; Cosme, K.; et al. CIGB-300, a novel proapoptotic peptide that impairs the CK2 phosphorylation and exhibits anticancer properties both in vitro and in vivo. *Mol. Cell. Biochem.* **2008**, *316*, 163–167. [CrossRef] [PubMed]
17. Perea, S.E.; Perera, Y.; Baladrón, I.; González, L.; Benavent, F.; Fariña, H.G.; García, I.; Rodríguez, A.; Reyes, V.; García, Y.; et al. CIGB-300: A promising anti-casein kinase 2 (CK2) Peptide for Cancer Targeted Therapy. In *Protein Kinase CK2 Cellular Function in Normal and Disease States. Advances in Biochemistry in Health and Disease*; Ahmed, K., Issinger, O.G., Szyszka, R., Eds.; Springer: Cham, Switzerland, 2015; Volume 12. [CrossRef]
18. Prins, R.; Burke, R.; Tyner, J.; Druker, B.J.; Loriaux, M.M.; Spurgeon, S.E. CX-4945, a selective inhibitor of casein kinase-2 (CK2), exhibits anti-tumor activity in hematologic malignancies including enhanced activity in chronic lymphocytic leukemia when combined with fludarabine and inhibitors of the B-cell receptor pathway. *Leukemia* **2013**, *27*, 2094–2096. [CrossRef] [PubMed]
19. D´Amore, C.; Borgo, C.; Sarno, S.; Salvi, M. Role of CK2 inhibitor CX-4945 in anti-cancer combination therapy—Potential clinical relevance. *Cell. Oncol.* **2020**, *43*, 1003–1016. [CrossRef] [PubMed]
20. Wu, B.; Zhang, B.; Li, B.; Wu, H.; Jiang, M. Cold and hot tumors: From molecular mechanisms to targeted therapy. *Sig. Transduct. Target. Ther.* **2024**, *9*, 274. [CrossRef] [PubMed]
21. Decraene, B.; Yang, Y.; De Smet, F.; Garg, A.D.; Agostinis, P.; De Vleeschouwer, S. Immunogenic cell death and its therapeutic or prognostic potential in high-grade glioma. *Genes Immun.* **2022**, *23*, 1–11. Correction in *Genes Immun.* **2022**, *23*, 244. https://doi.org/10.1038/s41435-021-00161-5. [CrossRef] [PubMed]
22. Galluzzi, L.; Vitale, I.; Warren, S.; Adjemian, S.; Agostinis, P.; Martinez, A.B.; Chan, T.A.; Coukos, G.; Demaria, S.; Deutsch, E.; et al. Consensus guidelines for the definition, detection and interpretation of immunogenic cell death. *J. Immunother. Cancer* **2020**, *8*, e000337. Correction in *J. Immunother. Cancer* **2020**, *8*, e000337corr1. https://doi.org/10.1136/jitc-2019-000337. [CrossRef] [PubMed]
23. Kim, Y.H.; Kwak, M.S.; Lee, B.; Shin, J.M.; Aum, S.; Park, I.H.; Lee, M.G.; Shin, J.-S. Secretory autophagy machinery and vesicular trafficking are involved in HMGB-1secretion. *Autophagy* **2021**, *17*, 2345–2362. [CrossRef] [PubMed]
24. Vázquez-Blomquist, D.; Ramón, A.C.; Rosales, M.; Pérez, G.V.; Rosales, A.; Palenzuela, D.; Perera, Y.; Perea, S.E. Gene expression profiling unveils the temporal dynamics of CIGB-300-regulated transcriptome in AML cell lines. *BMC Genom.* **2023**, *24*, 373. [CrossRef] [PubMed]
25. Chen, G.; Li, J.; Ochani, M.; Rendon-Mitchell, B.; Qiang, X.; Susarla, S.; Ulloa, L.; Yang, H.; Fan, S.; Goyert, S.M.; et al. Bacterial endotoxin stimulates macrophages to release HMGB-1 partly through CD14- and TNF-dependent mechanisms. *J. Leukoc. Biol.* **2004**, *76*, 994–1001. [CrossRef] [PubMed]
26. Rodríguez-Ulloa, A.; Ramos, Y.; Sánchez-Puente, A.; Perera, Y.; Musacchio-Lasa, A.; Fernández-De-Cossio, J.; Padrón, G.; López, L.J.; Besada, V.; Perea, S.E. The combination of the CIGB-300 anticancer peptide and cisplatin modulates proteins related to cell survival, DNA repair and metastasis in a lung cancer cell line model. *Curr. Proteomics.* **2019**, *16*, 338–349. [CrossRef]

27. Perera, Y.; Costales, H.C.; Diaz, Y.; Reyes, O.; Farina, H.G.; Mendez, L.; Gómez, R.E.; Acevedo, B.E.; Gomez, D.E.; Alonso, D.F.; et al. Sensitivity of tumor cell towards CIGB-300 anticancer peptide relies on its nucleolar localization. *J. Pept. Sci.* **2012**, *18*, 215–223. [CrossRef] [PubMed]
28. Pierre, F.; Chua, P.C.; O'Brien, S.E.; Siddiqui-Jain, A.; Bourbon, P.; Haddach, M.; Michaux, J.; Nagasawa, J.; Schwaebe, M.K.; Stefan, E.; et al. Pre-clinical characterization of CX-4945, a potent and selective small molecule inhibitor of CK2 for the treatment of cancer. *Mol. Cell. Biochem.* **2011**, *356*, 37–43. [CrossRef] [PubMed]
29. Rosales, M.; Rodríguez-Ulloa, A.; Pérez, G.V.; Besada, V.; Soto, T.; Ramos, Y.; González, L.J.; Zettl, K.; Wiśniewski, J.R.; Yang, K.; et al. CIGB-300-regulated proteome reveals common and tailored response patterns of AML cells to CK2 inhibition. *Front. Mol. Biosci.* **2022**, *9*, 834814. [CrossRef] [PubMed]
30. Villamaña, L.; Martínez-Escardó, L.; Arús, C.; Yuste, V.J.; Candiota, A.P. Successful Partnerships: Exploring the Potential of Immunogenic Signals Triggered by TMZ, CX-4945, and Combined Treatment in GL261 Glioblastoma Cells. *Int. J. Mol. Sci.* **2021**, *22*, 3453. [CrossRef] [PubMed]
31. Venereau, E.; Casalgrandi, M.; Schiraldi, M.; Antoine, D.J.; Cattaneo, A.; De Marchis, F.; Liu, J.; Antonelli, A.; Preti, A.; Raeli, L.; et al. Mutually exclusive redox forms of HMGB-1 promote cell recruitment or proinflammatory cytokine release. *J. Exp. Med.* **2012**, *209*, 1519–1528. [CrossRef] [PubMed]
32. Yang, H.; Lundbäck, P.; Ottosson, L.; Erlandsson-Harris, H.; Venereau, E.; Bianchi, M.E.; Al-Abed, Y.; Andersson, U.; Tracey, K.J. Redox modifications of cysteine residues regulate the cytokine activity of HMGB-1. *Mol. Med.* **2021**, *27*, 58. [CrossRef] [PubMed]
33. Kazama, H.; Ricci, J.E.; Herndon, J.M.; Hoppe, G.; Green, D.R.; Ferguson, T.A. Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of High-Mobility Group Box-1 protein. *Immunity* **2008**, *29*, 21–32. [CrossRef] [PubMed]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.