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Oxidative stress is widespread and entwined with pathological processes, yet its linkage to adaptive immunity remains elusive. Reactive carbonyl (RC) adduction, a common feature of oxidative stress, has been shown to target proteins to the adaptive immune system. Because aldehydes are important mediators of carbonylation, we explored the immunomodulatory properties of model Ags modified by common bioactive aldehyde by-products of oxidative stress: 4-hydroxy-2-nonenal, malondialdehyde, and glycolaldehyde. Ag modification with all three aldehydes resulted in Ag-specific IgG1-dominated responses in adjuvant-free murine immunizations in an RC-dependent manner. The central role of RCs was confirmed, as their reduction into nonreactive groups abrogated all adaptive responses, despite the presence of other well-known aldehyde-driven adducts such as N\(^{-}\)-carboxymethyllysine and glycolaldehyde–pyridine. Moreover, Ag-specific Ab responses robustly correlated with the extent of RC adduction, regardless of the means of their generation. T cell responses mirrored the Th2-biased Ab isotypes by Ag-specific splenocyte production of IL-4, IL-5, and IL-13, but not IFN-\(\gamma\). The RC-induced Th2 response was in sharp contrast to that induced by Th1/Th2 balanced or Th1-biasing adjuvants and was maintained in a range of mouse strains. In vitro studies revealed that RC adduction enhanced Ag presentation with Th2 polarization in the absence of conventional dendritic cell activation. Taken together, these data implicate commonly occurring RC as an important oxidation-derived Th2 immunomodulatory damage-associated molecular pattern with potentially important roles in health and disease. The Journal of Immunology, 2011, 187: 000–000.

In addition to protecting the host from infection, the immune system is involved in general homeostasis, exemplified by roles played by the innate immune system during sterile tissue stress and injury (1). It has therefore been proposed that the immune system can be alarmed by evolutionarily conserved endogenous structures termed damage-associated molecular patterns (DAMPs), which either do not occur in or are not released by, healthy tissues (2). Since the coinage of this term subsequent to that of pathogen-associated molecular patterns (3), several candidates have been proposed to act as DAMPs including those involved in the initiation of necrosis-induced inflammation (1). Although the list is expanding, other classes of DAMPs specific to homeostatic disturbances, such as commonly occurring oxidative stress, await further elucidation.

Oxidation is both harnessed and tightly controlled in biological systems, but when present in excess it can lead to oxidative stress, a common source of tissue and cellular injury (4, 5). Carbohydrate and lipid oxidation (glycoxidation and lipoxidation) generates a variety of chemical species, of which aldehydes are a highly reactive by-product (6). In addition to direct toxicity, aldehydic modification of biomolecules, particularly of proteins, gives rise to a multitude of adducts that are increasingly implicated in the pathology of a wide range of human diseases with prominent oxidative components (4–6). For many such conditions, including atherosclerosis, diabetes, Alzheimer’s disease, and alcohol liver disease, there exists a plethora of evidence in support of a critical role for innate immune engagement by lipoxidation- and glycoxidation-derived products (5), in particular aldehyde-modified proteins and phospholipids (7, 8). Such extensive, and in many cases proinflammatory, involvement of innate immunity is likely to provide fertile ground for the elicitation of adaptive immune responses. In support of this, T cell and B cell responses targeted to both modified and unmodified endogenous Ags have been well documented in oxidation-driven pathologies (9), exemplified by autoantigen and antiadduct Abs observed in atherosclerosis and alcohol liver disease (10, 11). However, far less is known about the molecular and immunological pathways by which these adaptive responses are initiated.

Reactive carbonyl (RC) adduction to proteins is generally considered a hallmark of direct or aldehyde-mediated oxidative stress (12). RCs were first implicated as the active moieties in driving adaptive immune responses against model Ags treated with glycolaldehyde (GA), a product of glycoxidation and the myeloperoxidase/H\(_2\)O\(_2\)/chloride pathway (13, 14), and NaOH, a common oxidizing agent (15). We subsequently extended this observation by demonstrating a role for formaldehyde-generated RCs in an exaggerated Th2-biased immune response implicated in vaccine hypersensitivity (16). Similar to GA, 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) are among the most common bioactive aldehyde products of oxidative stress (17), whose adducts have been implicated in various immune pathologies (5, 18) and linked to induction of adaptive immunity (19, 20). Because HNE and MDA attack on proteins can result...
in RC addition (21, 22), we investigated the significance of such structures and confirm here that RCs are indeed the central immune-active moieties via which these aldehydes modulate Ag immunogenicity. Furthermore, we show that RCs induce a profound Th2 biasing effect regardless of their mode of generation. Taken together, these data underscore the importance of the RC group as the main immune-active moiety of adducted structures on oxidatively stressed Ags and highlight their role in the Th2 polarization of Ag-specific immune responses.

Materials and Methods
Mice
Female BALB/c, 129S6/SvEv, CBA, C57BL/6, and DO.11.10 × SCID mice were bred and maintained in accredited University of Oxford facilities and used for experimental procedures at ages 6–9 wk. All procedures were authorized by institutional ethical review and conducted in accordance with the U.K. Home Office Animals (Scientific Procedures) Act 1986.

Protein modification
Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Protein Ags were either cleared of endotoxin using Detoxi-Gel endotoxin removing gel (Pierce, Rockford, IL) or purchased as an endotoxin-free product (EndoGrade OVA; Hyglos). The endotoxin levels were externally tested (Lonza, Verviers, Belgium) and reported as ranging from very low (≤0.2 EU/ml) to undetectable. For aldehyde modification, OVA or hen egg lysozyme (HEL; 0.25–0.5 mg/ml) were incubated with 20 mM GA, MDA [released from bis salt according to the method of Kikugawa and Ido (23)], or HNE (Axxora, San Diego, CA) solutions in LPS-free PBS (Life Technologies, Carlsbad, CA) pH 7.4 for 3 h at 37°C. For dose-response experiment, OVA was modified with 2 or 20 mM GA or formaldehyde (FA). Unbound aldehydes were removed by desalting via Amicon Ultra or Microcon filters (Millipore). To reduce RCs, 20–50 mM NaBH4 was used either at the time or after aldehyde treatment followed by desalting as above. For oxidation, 0.25–0.5 mg/ml OVA was incubated with 1 mM NaIO4 in sodium acetate buffer pH 4.5 at 4°C in the dark for 90 min, followed by desalting and reduction as above. The protein concentration in samples was measured using a BCA Protein assay (Pierce, Rockford, IL).

Mass spectrometry and carbonyl assays
Protein samples for mass spectrometry were extensively buffer exchanged into dH2O and then acidified with formic acid before being purified with a C4 reverse-phase Ziptip (Millipore, Bedford, MA). The eluted samples were vacuum-dried and then reconstituted in acetonitrile before injection into an LTQ Orbitrap mass spectrometer (Thermo Scientific, Hertfordshire, U.K.). All data were analyzed with XCalibur Software (Thermo Scientific).

RC measurement
DNPH-based colorimetric and ELISA assays were carried out as published previously (16). Briefly, for ELISA, protein samples were reacted with 10 mM DNPH solution in 2 M HCl for 45 min followed by coating on ELISA plates (Greiner bio-one, Frikenhausen, Germany) overnight in pH 8.5 NaHCO3 buffer and subsequent detection of DNPH-tagged RCs using...
a biotinylated anti-DNPH Ab (Molecular Probes, Eugene, OR) (16). Colorimetric detection of tagged DNPH was used for quantitative measurements as described previously (16) using molar absorption coefficient of DNPH (22,000 M⁻¹cm⁻¹) in reacted samples.

**Immunization**

Six- to nine-week-old mice were immunized s.c. in the flank using 10–50 μg of unmodified or modified proteins in 100 μl LPS-free PBS (Life Technologies) or with unmodified protein adjuvanted 1:1 in 50 μl Imject Alum (Pierce) containing 2 mg aluminum hydroxide, 50 μl Freund’s complete adjuvant (FCA; Sigma-Aldrich, St. Louis, MO) 1:1, or 20 μg ODN1826 CpG oligonucleotide (InvivoGen, San Diego, CA) where stated. In some experiments, mice were further boosted with 2–50 μg of either unmodified or modified adjuvant-free protein preparations between week 6 and 12 postpriming as indicated. Mice were bled prior to and after immunization and sacrificed for final blood samples and spleen harvests.

**Ab ELISA and titer calculation**

Sera from immunized mice were serially diluted, starting at 1/20, on ELISA plates (Greiner bio-one) coated with 5–10 μg/ml unmodified OVA or HEL, and Ag-specific isotype reactivity was detected using appropriate anti-mouse isotype HRP conjugates (Total IgG, Serotec, Oxford, U.K.; IgG1 and IgG2a, BD Biosciences, Oxford, U.K.; IgG2c, GeneTex). Plates were developed using a TMB (3,3′,5,5′-tetramethylbenzidine) substrate (Pierce) and absorbance (A₄₀₅) determined using a SpectraMax M5 microplate reader (Molecular Devices, Berkshire, U.K.). End-point titers were extrapolated, using cutoff points determined based on preimmune sera reactivity activity means at 1/20 ≥ 3 SD, from sigmoidal dose-response curves (4PL or SPL) upon log transformation of data in Prism software version 5.0 (GraphPad Software) and expressed as reciprocal titers. For dose-response analysis, OD values at a serum dilution of 1/100 were normalized to the detection limit of the assay ranging from 3 to 5 pg/ml.

**Splenic cytokine secretion**

Splenocytes were harvested from immunized mice at indicated time points, cultured at the density of 2 × 10⁶/ml in 24-well plates (Corning) in the absence or presence of 50 μg/ml unmodified Ag. Supernatants were collected at days 1–5 and frozen at −80°C. Cytokines were quantified using Bio-Plex as specified earlier, and for FACS analysis directly fluorochrome labeled Bio-Plex mouse cytokine kits (BioRad, Herts, U.K.) acquired and quantified on a Luminex 100 reader (Luminex, Austin, TX) according to the manufacturer’s instructions. Wells without Ag had cytokine levels below the detection limit of the assay ranging from 3 to 5 pg/ml.

**Dendritic cell pulsing and T cell proliferation**

Dendritic cells (DCs) were purified from BALB/c spleen by enzymatic digestion and density gradient centrifugation as published previously (24), followed by CD11c MACS positive selection (Miltenyi Biotec, Surry, U.K.) achieving purities ≥ 95% CD11c⁺MHC II⁺. Purified DCs were then pulsed with native or modified OVA at 100 μg/ml for 3 h at 37°C for Ag presentation assays or 12 h at 37°C for cytokine production and cell surface analysis by FACS. CD4⁺ T cells were isolated from DO11.10 SCID mice by MACS negative selection (Miltenyi Biotec) and labeled with 2.5 μM CFSE (Invitrogen, Paisley, U.K.). T cells (5 × 10⁵) were then coincubated with 1 × 10⁶ pulsed DCs (3 h) for a further 60–72 h. Supernatants were collected for cytokine analysis, and CD3⁺ T cell proliferation was assessed by flow cytometric CFSE dilution analysis using FACS-Calibur (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR). Immature DCs (bone marrow-derived dendritic cells; BMDCs) were derived by cultivating murine bone marrow in the presence of 20 ng/ml rGM-CSF (R&D, Abingdon, U.K.) for 6 d (adapted from Ref. 25) to ≥ 85% CD11c⁺CD11b⁺ purity before being pulsed and characterized as described for splenic dendritic cells (SPDCs). Cytokine analysis was performed using Bio-Plex as specified earlier, and for FACs analysis directly fluoresceinated Abs (BD Biosciences; Serotec, Oxford, U.K.) were employed using CellQuest software (BD Biosciences).

**Gel electrophoresis**

One to five micrograms of native or modified HEL or OVA was prepared and loaded onto Novex minigels (Invitrogen) and run under reducing conditions according to the manufacturer’s protocol. Gels were subsequently stained using a silver-staining kit (Amersham International, Buckinghamshire, U.K.) or SimplyBlue SafeStain (Invitrogen) following the manufacturer’s protocols.

**Statistical analysis**

Statistical analyses were performed using Prism software version 5.0 (GraphPad Software). In vivo data were handled using non-parametric tests (Mann–Whitney U test; Kruskal–Wallis test and Dunn post hoc test for multiple comparisons; Spearman test for correlation). All other data were tested using Student t test or one-way ANOVA and appropriate post hoc tests (Dunnett test for multiple comparisons with control). Means and standard deviations are presented for normally distributed data sets, whereas for in vivo data, no assumption of normality was made and data are depicted by median and range.

**Results**

**RC addition to proteins by common aldehyde-by-products of oxidative stress enhances Ag immunogenicity**

Adduction of RCs to Ags (Fig. 1A, reaction 1) by GA, FA, and NaO₄ treatment has previously been shown to result in enhanced Ag immunogenicity (15, 16). HNE and MDA are among the most studied aldehyde-by-products of oxidative stress (5) and have been identified on a Luminex 100 reader (Luminex, Austin, TX) according to the manufacturer’s instructions. Wells without Ag had cytokine levels below the detection limit of the assay ranging from 3 to 5 pg/ml.

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implicated in immune modulation (19, 20), but the chemical nature of the immune-modulating activity remains inconclusive. Because RCs are commonly adducted by HNE and MDA (Fig. 1B) (21, 22), we investigated their effects on model Ag immunogenicity. Treatment of HEL with HNE and MDA resulted in significantly increased RC content that was comparable with that adducted by GA (Fig. 1C), as determined by DNPH-based ELISA (Fig. 1A, reaction 2) (26). Subcutaneous administration to BALB/c mice of RC-adducted Ag in the absence of adjuvant resulted in enhanced immunogenicity of HNE, MDA, and GA-modified HEL, as determined by naïve Ag-specific IgG responses, comprising high IgG1 titers but with undetectable or low titers of IgG2a across groups (Fig. 1C).

RCs can be eliminated via reduction into less reactive alcohol groups by borohydrides such as NaBH4 (Fig. 1A, reaction 3) (27, 28). Using this method, we eliminated the RCs from HNE- and MDA-treated HEL (Fig. 1B, 1C), and, similar to GA, this reduced all HEL immunogenicity to the levels elicited with unmodified HEL (Fig. 1C). These results suggested that RC may be the central immune-activating moiety, irrespective of the diversity of HNE, MDA, or GA adducts present (13, 21, 22). This was consistent with the observation that the median size of Ab responses appeared to be related to the HEL RC content (Fig. 1C). To investigate this further, we adducted RCs to a second model Ag, the glycoprotein OVA, by aldehyde treatment (2 or 20 mM FA or GA) or by NaIO4 oxidation of the glycan (16, 27, 29). After adjuvant-free immunization of BALB/c mice, OVA-specific total IgG responses, pooled irrespective of the method of modification, correlated robustly with the moles of adducted RCs on OVA (p = 0.71, p = 0.036; Fig. 1D). Given that these methods can also generate distinct arrays of other products, such a correlation confirms the data for HEL (Fig. 1C) and highlights the importance of RC in the observed Ag immunogenicity.

RCs but not other oxidation-related adducts account for increased protein immunogenicity

Apart from RC-containing derivatives, aldehyde attack on proteins can give rise to a multitude of other adducts (6), some of which, such as Nε-carboxymethyllysine (CML) (Fig. 1A, reaction 4) (13), have been proposed to possess immune-activating properties (30, 31). Selective elimination of RCs from aldehyde–protein adducts through borohydride reduction [Fig. 1A (reaction 3), 1B ("Reduced Adducts") serve as a reliable control in the investigation of immune-activating roles played by RC moieties (15, 16). These

![Image 1](https://www.jimmunol.org/)

**FIGURE 4.** In vivo Th polarization profiles of RC-adducted Ag. A, BALB/c (n = 5) were immunized with 10 μg HEL, unmodified (Unmod), GA-treated (GA), or GA-treated and reduced (GA-R), and boosted once with 2 μg HEL 6 wk later. At week 12, splenocyte cultures were pulsed ex vivo with HEL, and day 1 and 5 supernatants were assayed for cytokines using the Lumixx multiplex system. B, IL-4 and IFN-γ secretion from the HEL/FCA immunized mice, run in the same experiment as A, is compared with other groups. C, Splenocyte cytokine profile of HNE and MDA modified HEL, or HEL in alum, run as in A. D, Splenocyte cytokine profile of 129S6/SvEv or C57BL/6 mice (n = 4 and 5), immunized and boosted with 10 or 50 μg GA-treated HEL, respectively. Box and whiskers are minimum and maximum. Data are representative of at least two independent experiments. *p ≤ 0.05, **p ≤ 0.01.
reducing agents, when applied at the time of aldehyde reaction with protein (frequently with the ε-amine of lysine), drive the reactions toward alcohol groups (Fig. 1A, reaction 3) (27), which prevents the development of other end products (Fig. 1A, reaction 4). However, reduction post-aldehyde treatment should only selectively reduce the aldehydic RC groups, leaving other derived stable products, such as CML, intact. To test this, we first treated HEL with GA and then reduced the resulted adducts using NaBH4 and analyzed the samples by mass spectrometry. We identified three major GA–lysine derived adducts: aldoamine, CML, and GA–pyridine (Fig. 2A, “HEL/GA”) (13, 27, 32). Upon reduction, the RC-containing aldoamine peak was replaced by the hydroxethyl alcohol form, whereas the CML and GA–pyridine peaks remained intact (Fig. 2A, “HEL/GA-R”). Adjuvant-free immunization of BALB/c mice with these samples resulted in HEL-specific IgG1 dominant responses only in the sample containing RC (aldoamine) and not hydroxethyl, carboxymethyl, and GA–pyridine lysine derivatives (Fig. 2A, 2B). These results confirm that RCs are the major immune-modulating adducts of aldehyde treatment.

RCs can mediate intermolecular cross-linking, potentially giving rise to multimers that may contribute to enhanced immunogenicity (33). However, it was shown previously that the monomeric RC-bearing fraction of Ag remained as immunogenic as the non-fractionated material containing multimers (15). In this study, HEL reduced subsequent to GA treatment still contained comparable levels of cross-linked protein species but, similar to unmodified HEL, it only induced low Ab titers (Fig. 2B). Furthermore, RC adduction of OVA by NaIO4 oxidation rendered the protein immunogenic despite minimal cross-linking visualized by sensitive silver staining (Fig 2C), and a subsequent reducing step significantly reduced the Ab responses to low titers (Fig. 2C). These results highlight the central role of RC moieties in eliciting observed adaptive humoral responses, independent of the adduct types or of cross-linking.

**RCs drive robust Th2-biased responses independently of mouse genetic background**

The Ab responses we observed were all of dominant IgG1 isotypes (Figs. 1, 2), indicative of Th2-biased immunity (34). To explore this further, we amplified the humoral responses with a more extensive prime/boost immunization of BALB/c mice and used GA treatment of Ag as a particularly efficient means of RC adduction (Fig. 1B, 1C). As before, RC-adducted HEL consistently elicited high titers of IgG1 but only modest IgG2a titers, similar to Ag formulated in an aluminum-based adjuvant (alum) (Fig. 3A). By contrast, HEL administered in FCA induced comparable levels of IgG1 to RC-adducted Ag but significantly higher IgG2a titers (Fig. 3A). Certain strains of inbred mice have been attributed with inherent adaptive immune biases, with BALB/c generally regarded as Th2-biased (35). To address this, we compared RC-adducted HEL to HEL adjuvanted in CpG oligodeoxynucleotide (CpG...
ODN) in a more balanced strain of mice, 129S6/SvEv (36). As before, RC adduction induced a robust IgG1-dominated response in contrast with the Th1-biasing CpG ODN, which exhibited the expected IgG2a-biased response (Fig. 3B). Finally, the RC-driven Th2 isotype profile was further reproduced in mouse strains generally considered to elicit an intrinsically Th1-biased response, CBA and C57BL/6 (36) (Fig. 3C).

Parallel studies of T cell responses confirmed the RC-driven Th2 bias. Splenocytes from BALB/c mice immunized with GA-treated HEL, but not the unmodified or GA-treated and reduced samples, responded to ex vivo restimulation by secreting significant amounts of IL-2, IL-3, IL-4, IL-5, IL-6, and IL-13, but only trace levels of TNF-α and no detectable IFN-γ (Fig. 4A). This was in contrast to the FCA-adjuvanted HEL, which showed dominant IFN-γ with minimal IL-4 secretion in the same mouse strain (Fig. 4B). Furthermore, splenocytes from BALB/c mice immunized with MDA or HNE-treated HEL showed the same Th2 bias in their cytokine secretion, similar to that of alum-adjuncted HEL immunized mice (Fig. 4C). Immunization of the more balanced or Th-1 biased mouse strains 129S6/SvEv and C57BL/6, respectively, with HEL–GA similarly elicited IL-5 and IL-13 dominant responses with minimal TNF-α and IFN-γ (Fig. 4D). These data confirm the profound Th2 biasing property of RC adduction on Ags. To explore this effect at the cellular level, we next investigated RC–Ag–induced immune modulation in an in vitro Ag presentation model.

**RC adducts enhance Ag presentation and T cell proliferation with a Th2 bias in the absence of conventional costimulation**

Aldehyde-treated proteins have been reported to be targeted for increased uptake and processing (37, 38), resulting in enhanced T cell proliferation (39), in an RC-dependent manner (15). However, neither the effects of RC-adducted Ag on DC activation and maturation nor their subsequent polarization of T cells have been studied. To investigate this, freshly isolated murine SPDCs were pulsed with unmodified, RC-adducted OVA via GA treatment, or reduced GA-treated OVA then tested for their ability to induce proliferation of DO11.10 (OVA-TCR transgenic) CD4+ T cells. Significantly enhanced OVA-specific T cell division, as demonstrated by flow cytometric analysis of CFSE-labeled cells, was observed for RC-containing samples above that of unmodified or GA-treated and reduced preparations (Fig. 5A), coordinate with levels of IL-2 secretion (Fig. 5B). Significant IL-4 and a trend for increased IL-13 secretion were induced by RC-adducted OVA, whereas IFN-γ was dominantly induced with OVA formulated in CpG ODN (Fig. 5B). These opposing cytokine profiles corroborate our earlier immunization results (Figs. 3, 4) lending further support to the Th2-polarizing nature of RC adduction.

To investigate whether RC adducts mediate DC activation, SPDCs were exposed to modified or unmodified OVA preparations or OVA formulated with LPS or CpG ODN and analyzed by flow cytometry for surface markers and multiplex assay for cytokine release. Whereas OVA administered with LPS or CpG ODN resulted in significant upregulation of CD40, CD80, and CD86, no detectable increases were seen for the RC-adducted sample, which displayed a phenotype similar to that of unmodified and GA-treated and reduced OVA (Fig. 5C). Similarly, OVA/LPS or CpG ODN elicited high levels of IL-1β, IL-6, IL-12 p70, TNF-α, and KC, whereas RC adduction was associated with weak DC cytokine responses, indistinguishable from unmodified and GA-treated and reduced OVA (Fig. 5D).

SPDCs may be partially activated as a result of ex vivo manipulation, probably explaining the high level of MHC class II expression across all groups (Fig. 5C, data not shown). Because it is plausible that partial activation may mask subtle SPDC responses to RC-adducted Ag, we stimulated immature BMDCs with unmodified or GA-treated RC-adducted HEL and OVA. As with the SPDCs, we found no significant upregulation of CD40, OX40L, CD80, or CD86 or cytokine release by RC-adducted compared with unmodified proteins (Fig. 6A, 6B, data not shown). These data collectively suggest a lack of requirement for conventional activation of APCs for their role in RC-driven Th2 immune enhancement.

**Discussion**

Oxidative stress is thought to be a major contributor to a diverse range of human pathologies, yet despite its interactions with innate immunity, the links to adaptive immune responses have remained ill-defined. Aldehyde-modified proteins, commonly occurring under oxidative stress, have long been reported to be targeted to a range of immune-related cells (37–39) and to exhibit immune-modulating activities (40, 41). Allison and Fearon (15) reported...
that aldehydic RC is the structure through which GA and NaIO₄ treatment of Ags enhanced immunogenicity of model Ags, and this was further extended in our laboratory to account also for formalin-driven immune modulation in a vaccine context (16). In the current study, we have extensively characterized RC as a key Th2-biasing DAMP, accountable for the immune-enhancing properties of important aldehyde by-products of oxidative stress including HNE, MDA, and GA.

Lipoxidation-derived HNE and MDA adducts have long been identified as targets for adaptive arms of immunity in oxidative-related pathologies (42, 43). In the absence of clear mechanistic pathways, the formation of neo-epitopes introduced by adduction of aldehydes has been a popular concept in the study of adaptive immune responses induced by oxidative stress (19, 41). Emphasis has thus been placed on the altered antigenicity of adducted Ags rather than modified immunogenicity. Accordingly, in many experimental models, the detection and analysis of intrinsic immune-enhancing signals has been masked or blurred by the formulation of aldehyde–Ag adducts in strong exogenous adjuvants and the frequent use of reducing conditions to increase reaction efficiencies (19, 41, 44). Increased immunogenicity of MDA adducts has been reported in a few publications using nonreduced and non-adjuvanted samples, but the immunomodulatory structures were not elucidated, and the role of RC was not investigated (20, 39, 40, 45). By contrast, our data underscore the intrinsic RC-driven immune-enhancing properties of HNE and MDA protein adducts by 1) excluding extrinsic adjuvants; 2) monitoring adaptive responses against the native B cell and T cell epitopes; 3) demonstrating that the major non-RC adducts present in the reduced preparations are nonimmunogenic. This latter point is important because aldehyde attack on molecules can result in an array of protein adducts, depending on the reaction conditions, some of which may not contain RCs (6). The complex nature of these adducts has been particularly well studied in the context of advanced glycation end products (AGEs) for their contribution to AGE-related pathologies such as diabetes (46). However, in our hands, well-known AGE derivatives CML and GA–pyridine, implicated in enhanced presentation to T cells (31), failed to increase Ag immunogenicity in vivo in the absence of RCs, although harsher oxidation of protein with NaIO₄ did result in some residual immunogenicity independent of RCs (Fig 2C, “Ox-R”), which may potentially be explained by effects of Ag aggregation.

Aldehyde-modified proteins have been shown to be targeted to APCs, resulting in increased uptake and presentation (37–39), with RC suggested as an active ligand in this process (15). However, the impact of RC-bearing Ag on APC activation and consequent cell polarization has remained unexplored. Our results confirm the critical role of RC in enhancing T cell proliferative responses to the aldehyde-adducted Ag and demonstrate the Th2-polarizing properties of RC in vitro. Although this implies a central role for APCs in conferring the Th2 polarizing effects of RC adducts that we observed in vivo, we were not able to detect any conventional signs of DC activation, as determined by surface markers and cytokine secretion. Such a marked lack of conventional DC activation coupled with Th2 proliferative responses is not rare and is frequently described as being a “default pathway” or in terms of “alternative activation” (47). One potential marker for Th2-type signaling on DCs, OX40L (48), was not detected on our RC–Ag-pulsed BMDCs. Although other costimulation pathways (48) remain to be explored, it is conceivable that the well-characterized increased uptake of aldehyde adducts through scavenger receptors (37, 38, 45) may be sufficient for an enhanced Ag presentation (39), leading to Th2 immune induction as a result of failure to switch on Th1 pathways (47).

Our perception of oxidative damage and disease has substantially shifted toward a more immune-oriented perspective. Oxidative stress is a collective term implicating various reactions with distinct chemical modifications, which would inevitably interact, one way or another, with homeostatic maintenance. Although there is some evidence in the literature for other oxidation-derived immunomodulators (49), we provide robust evidence in this study that RCs are a major ubiquitous Th2-biasing oxidation-associated DAMP. From an evolutionary point of view, a selective advantage for RCs in bridging innate to adaptive immunity is not yet clear. An alternative nonselective explanation could be that a heightened state of oxidation, for example due to postindustrial changes in our diet and environment, can overwhelm the clearance and repair systems of innate immunity leading to unwanted enhanced Ag presentation and adaptive responses. Although there is some evidence to suggest that aldehyde/RC-driven Th2 responses can play both pathogenic (16) and protective roles (50, 51) depending on the context, a clearer perspective will await more detailed studies of relevant disease models.

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Disclosures
The authors have no financial conflicts of interest.

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role in diseases and therapeutic prospects for the inhibitors. 


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