Xanthine oxidoreductase is central to the evolution and function of the innate immune system

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The housekeeping enzyme xanthine oxidoreductase (XOR) has been studied intensively over the past 100 years, yet the complexity of its *in vivo* function is still poorly understood. A large body of literature focuses on the different catalytic forms of XOR and their importance in the synthesis of reactive oxygen and reactive nitrogen species, which are involved in many disease processes. More recently, various protective physiological roles of XOR have been recognized. We summarize for the first time that XOR is a component of the innate immune system. Because XOR is involved in multiple features of innate immunity we suggest that it is central to the evolution and function of this ancient defense system. We present evidence suggesting that XOR is a direct downstream target of the evolutionarily conserved Toll-like receptor–NF-κB pathway and discuss that numerous forms of post-translational modification of XOR could provide intrinsic molecular switches that make XOR an ideal component of various fast innate immune responses.

Xanthine oxidoreductase (XOR) is best known as an evolutionarily conserved housekeeping enzyme with a principal role in purine catabolism [1,2]. Humans with mutations in *XOR* suffer from xanthinuria, an autosomal-recessive disorder resulting in kidney stone formation and urinary tract disorders [3]. By generating mice with a targeted disruption of *XOR*, we discovered the additional role of XOR as an essential protein for milk fat droplet secretion from the lactating mammary gland [4]. This surprising function prompted us to review the complex role of XOR in mammals and throughout the animal kingdom. There is increasing evidence that XOR has additional physiological functions associated with its synthesis of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which have important roles in inflammation and host defense [5,6]. Here, we propose for the first time that XOR is an evolutionarily conserved component of the innate immune system.

XOR performs various cellular protective functions central to the innate immune system

In purine catabolism, XOR catalyzes the oxidative hydroxylation of hypoxanthine to xanthine and subsequently of xanthine to uric acid [1,2]. This, and presumably other, metabolic reactions of XOR have a far-reaching impact on cellular homeostasis, cellular protection from toxic compounds and also systemic protection, known as innate immunity. Uric acid and its oxidation product allantoin act as potent antioxidants and free radical scavengers, necessary to protect a cell from oxidative damage caused by numerous naturally occurring ROS and RNS [7–9]. XOR has, therefore, important functions as a cellular defense enzyme against oxidative stress. Interestingly, XOR itself contributes to the synthesis of numerous ROS and RNS [1,2,5,6]. XOR exists in two forms, as xanthine dehydrogenase (XD; EC 1.1.1.204), which is the primary gene product of XOR, and as xanthine oxidase (XO; EC 1.1.3.22), which is formed through post-translational modifications of XD. XD favors the cofactor NAD^+ as its primary electron acceptor, yet XO is unable to bind NAD^+ and uses O_2 as its electron acceptor. With both forms, but particularly with the XO form, numerous ROS and RNS are synthesized [1,2,5,6]. Consequently, the synthesis of both an antioxidant (uric acid) and numerous free radicals (ROS and RNS) makes XOR an important protective regulator of the cellular redox potential [10]. Moreover, XOR is also a potent detoxification enzyme, probably owing to its multifunctional enzymatic activities [11]. This detoxification function is not restricted to XOR but is rather generally found in the molybdopterin family of enzymes, including aldehyde oxidase (AO) [11]. In this context, AO is of particular interest because its amino acid identity and its intron–exon structure is similar to XOR, suggesting that both enzymes evolved through gene duplications [12]. Not surprisingly, XOR and AO have a large number of common substrates and similar expression patterns [12].

The innate immune system is an evolutionarily conserved, rapid, non-specific first-line defense mechanism that incorporates elements of cellular differentiation and protection against oxidative stress [13]. Innate immunity is composed of: (i) surface epithelia that provide local physical and molecular barriers, (ii) inflammatory

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reactions and the activation of conserved cell-signaling pathways, (iii) numerous systemic protective molecules and (iv) various phagocytotic cells. All of these components work together to resist and prevent the action of toxic molecules and the rapid spread of potentially fatal pathogens. The protective functions of XOR in innate immunity are, as at the cellular level, linked to its detoxification reactions, its synthesis of uric acid and, particularly, its synthesis of numerous ROS and RNS.

Uric acid not only acts as a cellular but also as a systemic antioxidant and free radical scavenger, besides being an anti-inflammatory effector with numerous protective roles in the body [14–16]. ROS and RNS perform, at low levels, numerous cellular and physiological functions as second messengers but, at high levels, can act as microbicidals [17–19] (Fig. 1). XOR generates the ROS superoxide anions (O_2^-), hydroxyl radicals ($OH^{\prime}$) and hydrogen peroxide (H_2O_2) [20]. Those ROS can generate an entire cascade of microbicidal reactions and products by participating in the synthesis of additional antimicrobial and cytotoxic molecules, thereby broadening their range of action [21]. RNS derive from the free radical nitric oxide (NO), a product of NO synthase as well as XOR [22]. NO itself is antimicrobial and cytotoxic, and it is further involved in the generation of an array of reactive molecules and even more potent antimicrobial substances, which makes NO a defensive molecule against various pathogens, tumor cells and alloantigens [23,24]. XO has also been implicated in protective antiviral responses by catalyzing the conversion of retinaldehyde to retinoic acid. Derivatives of retinoic acid can inhibit viral replication, thus potentially preventing the spread of viral diseases [25]. As we summarize here, XOR and its products are directly and/or indirectly involved in multiple features of innate immunity in mammals and, furthermore, have a conserved defense function throughout the animal kingdom.

XOR is expressed and secreted by numerous surface epithelia

Because most pathogens enter the body through surface membranes, epithelial tissues are an important component of the non-specific immune system. Surface epithelia have evolved many defense mechanisms, among which are the expression and secretion of numerous molecules. These molecules perform broad-spectrum antimicrobial functions by neutralizing and inactivating pathogens and/or by generating transient microbicidal and cytotoxic substances, such as ROS and RNS. As a housekeeping enzyme, XOR is presumably expressed in all cells, yet, high levels of constitutively expressed XOR, as well as AO, are found in many mammalian epithelial and capillary endothelial tissues of various organs [26–29]. The intensity of XOR activity appears to be organ-dependent [28,29]. Numerous pathogens, cytokines, as well as many forms of tissue damage, can rapidly induce additional expression and translation of XOR [5]. Although XD is the predominant XOR form found in normal cells and tissues, XO appears to have an important role in cell and tissue injuries [5,6]. Various forms of stimuli induce the conversion of the XD to the XO form, presumably resulting in intensive synthesis of ROS and RNS [5,6].

XOR and uric acid are also secreted on the surface of (mucosal) epithelial tissues, thereby expanding their protective function extracellularly. Uric acid is found in nasal fluid, saliva and many other body fluids [14,30,31]. Uric acid is also released from the heart and the microvascular endothelium was identified as its site of formation [32]. XOR itself is constitutively released by

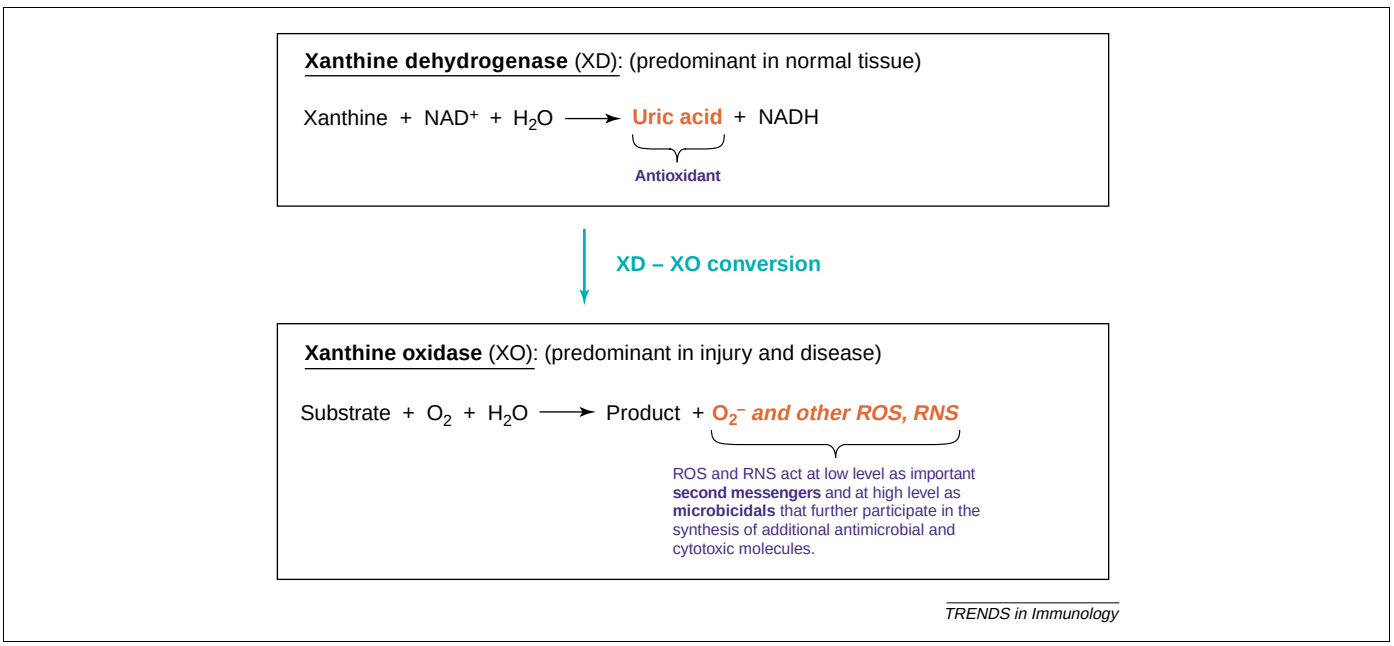

Fig. 1. XOR is a housekeeping enzyme with a role in purine catabolism, detoxification and the regulation of the cellular redox potential. XOR exists in two enzymatic forms, as a XD and as an XO. XD is the form that predominates in purine catabolism resulting in the synthesis of the antioxidant uric acid. The XO form is associated with the synthesis of large amount of ROS and RNS, which at low levels are important second messengers but at high levels have microbicidal action. The ability of XOR to rapidly convert from XD to XO under various forms of tissue injury and damage makes XOR an ideal component for a fast innate immune response. Abbreviations: NAD, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; RNS reactive nitrogen species; XD, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine oxidoreductase.

pulmonary microvascular endothelial cells and found on the outside surface of endothelial plasma membranes of various organs [33–35]. More importantly, XOR is secreted by the lactating mammary gland and the small intestine for an antimicrobial purpose [19,36,37]. Although the mammary gland and milk provide immunological protection for the offspring, the intestine needs an intensive immune defense owing to its expansive epithelial surface, its multitude of villi and narrow invaginations, its exposure to dietary antigens and its proximity to the colon. XOR activity was detected in enterocytes and in the mucous of duodenum [38]. Within the small intestine, XOR was detected in Paneth cells, which are specialized epithelial cells that secrete various antimicrobial molecules in responsonse to bacterial and other stimuli [39].

XOR is a mediator of infection and inflammation and interacts with NF-κB and activator protein-1 (AP-1)

Inflammatory reactions are an important part of innate immunity, comprising a sequence of events induced by various forms of tissue damage and infection. The inflammatory reaction results in the expression of various cytokines and XOR is stimulated by interferon-γ (IFN-γ), IFN-α, tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-3; some of these factors also initiate the conversion from the XD to the XO form [5,6]. Endothelial XOR was suggested to trigger a microvascular inflammatory response, leading to recruitment and activation of circulating neutrophils and trapping of circulating pathogens [6]. The recruitment of neutrophils and the activation of macrophages are induced by ROS that upregulate cell adhesion molecules [40,41]. ROS from XOR and NADPH oxidase mediate the expression of endothelial-cell surface P-selectin, one of the first cell-surface molecules to be expressed on the endothelial cell in response to inflammation [42].

Throughout evolution, pathogen-induced stimulation of the innate immune system relies on numerous conserved Toll-like receptors (TLRs), which initiate intracellular signaling cascades that subsequently activate the transcription factors NF-κB and AP-1 [43,44]. NF-κB controls a wide variety of immune-inducible antimicrobial molecules, including ROS and RNS, as well as cytokines and co-stimulatory molecules [45]. Binding sites for NF-κB and AP-1, as well as for response elements for cytokines, have been identified in the 5′ UTR (untranslated region) of the human *XOR* gene and both NF-κB and XOR are stimulated by bacterial lipopolysaccharids (LPS) *in vivo* [28,46,47]. This suggests that XOR is a downstream target of the TLR–NF-κB pathway and is directly activated by NF-κB and AP-1. Yet, both transcription factors are also stimulated by the redox potential of the cell and XOR might, therefore, also regulate the expression of NF-κB and AP-1 [48,49]. The application of an inhibitor for XOR leads to a dose-dependent inhibiton of NF-κB during hepatic ischemia-reperfusion, and AP-1 is downregulated following the synthesis of NO [50,51]. Consequently, we suggest intensive cross talk between XOR, the TLR–NF-κB pathway and the transcription factors NF-κB and AP-1.

XOR participates in systemic protection and detoxification

XOR activity and uric acid are generally found in the blood plasma of many mammalian species and levels are particularly high during numerous disease states [5]. XOR might participate in the systemic antimicrobial response of the innate immune system by providing an oxidative defense mechanism in the blood. XOR is reported to have a defense role in meningitis, malaria and in trypanosomiasis [15,52,53]. Serum XOR is predominantly in the XO form, as a result of serum proteases [54]. XOR and the synthesis of ROS have also been implicated in the activation of the complement system, an important feature of the systemic innate immune system [55,56].

XOR is highly expressed in the liver for purine catabolism and nitrogen elimination as well as for systemic detoxification. XOR and AO are both important for the biotransformation of numerous toxic compounds [11]. Yet, the liver is also an organ for systemic bacterial scavenging, inactivation of bacterial products and inflammatory mediator clearance and production [57]. Liver Kupffer cells are a major source of ROS and proinflammatory cytokines that are produced in response to LPS, making the liver a key organ for the killing of circulating microorganisms [58]. ROS generated by both XOR and NADPH oxidase-dependent pathways are important for the systemic killing of *Candida parapsilosis* by Kupffer cells and the inhibition of XO activity leads to significant reduction of hepatic killing of various bacterial strains [59–62]. Furthermore, the liver produces various antimicrobial serum proteins that are released in response to infection or inflammation and generally are known as the acute phase proteins. The way cytokines stimulate XOR activity is similar to an acute phase response, and XOR might also be considered to participate in this feature of innate immunity [5,53]. Several other tissues and organs upregulate XOR in response to systemic infections, including the spleen, which is the major organ for pathogenic scavenging [28,63].

XOR has an important role in phagocytotic killing

Innate and adaptive immunity rely on the activity of phagocytotic cells that release numerous molecules, including ROS, for the intracellular destruction of phagocytosed microorganisms and for extracellular toxic effects on pathogens and tumor cells. Several enzymes are responsible for the synthesis of ROS in phagocytes and experiments suggest that XOR also participates in the innate immune response of phagocytotic cells [18,21]. Animals infected with *Staphylococcus aureus*, *Salmonella typhimurium*, *Plasmodium berghei*, *Schistosoma mansoni*, Listeria, various viruses and Ehrlich hyperdiploid carcinoma cells, all show a marked increase in XO activity in phagocytotic cells. Most of the XOR activity found in these infected animals is in the XO form. The administration of XOR inhibitors to infected animals leads to a decrease in the bactericidal activity of phagocytes and a linear dose-increase in mortality. Administration of purified XO or xanthine, a XOR substrate, to infected animals, results in significant protection against bacterial infections [61,64,65]. Furthermore, XOR contributes to

host defense against *Burkholderia cepacia* and phagocytic destruction of *Candida parapsilosis* by murine peritoneal macrophages [66,67].

XOR participates in the innate immune system throughout the animal kingdom

XOR has general cellular protective functions, presumably present in all eukaryotic and most prokaryotic cells. Yet, there is evidence that its role in systemic protection and innate immunity is also conserved. Because innate immunity is evolutionarily ancient and preceded adaptive immunity [68], the protective role of XOR in the innate immune system of lower organisms might be particularly crucial for their defense and survival. Invertebrates possess an efficient innate immune system composed of cellular and humoral elements. The humoral defense includes antimicrobial peptides, the cascades that regulate coagulation and melanization of hemolymph, and the production of ROS and RNS [69]. The involvement of cytotoxic ROS and RNS in innate immunity has been studied intensively in *Drosophila* and other insects, as well as in bivalve mollusks [21,24,70,71]. Uric acid is known to occur in large amounts in *Drosophila* and a decline of this antioxidant with age could be associated with the loss of antioxidant potential in aging [72]. The melanization reaction is performed by many arthropods as part of the innate immune system and triggered by injury and recognition of microbial cell-wall composition. Melanization leads to the synthesis and deposition of melanin in wounded areas, resulting in wound healing, synthesis of toxic products (including ROS and RNS), sequestration, encapsulation and killing of invading microorganisms [73,74]. XOR and ROS have been implicated in the synthesis of melanin and it has been postulated that the melanization of skin and other tissues is also an important component of the innate immune system of vertebrates [75].

In mammals, XOR, as well as AO, is involved in numerous hepatic and epithelial detoxification reactions. In insects, insecticide resistance and insecticide detoxification are important host defense mechanisms, resulting in the upregulation of various metabolic enzymes. These enzymes are also involved in anabolism and catabolism of a multitude of endogenous compounds and the protection against oxidative stress [76]. *AO* is coamplified with the Culex mosquito insecticide resistance-associated *esterases* and its 5′ flanking region contains binding sites, known from other insecticide resistance genes [12,77]. As mentioned, the TLR–NF-κB-signaling cascade has a central role in innate immunity throughout the animal kingdom and crosstalk between XOR, ROS and NF-κB has been outlined.

The complex and versatile role of XOR, particularly in the mammalian innate immune system, could be linked to the unique rapid post-translational conversion from the XD to the XO form. Only mammalian XOR, but not XOR from chicken and *Drosophila*, can be converted from the XD to the XO form experimentally. XD is reversibly converted to XO by thiol oxidation or irreversibly by limited proteolysis [2,78]. The mechanism for this conversion might have evolved very recently. Phylogenetic analysis of the presence of various oxidases demonstrate that although the XD form of XOR is present throughout all vertebrates except reptiles, the XO form is only found in mammals. XOR in its XD form is abundant in insects, although crustacea and gastropoda seem to have much higher levels of AO than XOR. Neither XOR nor AO activity was detectable in Porifera, Coelenterata, Platyhelminthes, Nemathelminthes and Nemertea [79]. This suggests that the systemic protective role of XOR, and probably also of AO, is found predominantly in higher invertebrates and presumably, throughout all vertebrates, with a particular complex function in mammals. The possibility to rapidly, reversibly convert XOR from the XD to the XO form, might provide mammals with a powerful intrinsic switch, ideally suited as a component for a rapid, transient, innate immune response, independent of immunological memory. In general, most effector molecules of innate immunity need a fast conversion from a pre-existing, inactive to an active form, which is usually achieved by proteolytic processing of a precursor molecule [80]. Yet, other forms of post-translational activation of XOR could be of similar importance and the strong emphasis of the literature on the XD to XO conversion has been questioned [5,6]. XOR also exists in enzymatic inactive demolybdo and desulfo forms [5]. Experiments show that a desulpho–sulpho conversion of XOR is associated with an increase in XOR activity [5]. Moreover, activation of XOR by phosphorylation has been suggested [81]. It is possible that various forms of post-translational activation of XOR might act as switches for the complex role of XOR in numerous fast innate immune responses.

Conclusions

The ability of the multifunctional enzyme XOR to perform general detoxification reactions and additionally, to synthesize large amounts of the antioxidant uric acid, as well as ROS and RNS, makes it a versatile intra- and extra-cellular protective housekeeping enzyme and an important component of the innate immune system. XOR is involved in numerous features of mammalian innate immunity but, furthermore, its protective role appears to be evolutionarily conserved and downstream of the TLR–NF-κB pathway. Because numerous features of the innate immune system include oxidative defense and XOR and elements of cellular oxidative protection predated the existence of the innate immune system, we suggest that XOR is a central molecule in the evolution and function of this ancient defense system (Fig. 2). Because the innate immune response generates many signals that co-stimulate the adaptive immune system, it is possible that XOR also has a role in the crosstalk between innate and adaptive immunity.

As mentioned, we recently discovered that XOR also has an essential role in milk fat droplet secretion from the lactating mammary gland. How XOR and the innate immune system contributed to the evolution of the mammary gland, milk and the entire class mammalia, will be discussed in another communication (C. Vorbach and M. Capecchi, unpublished).

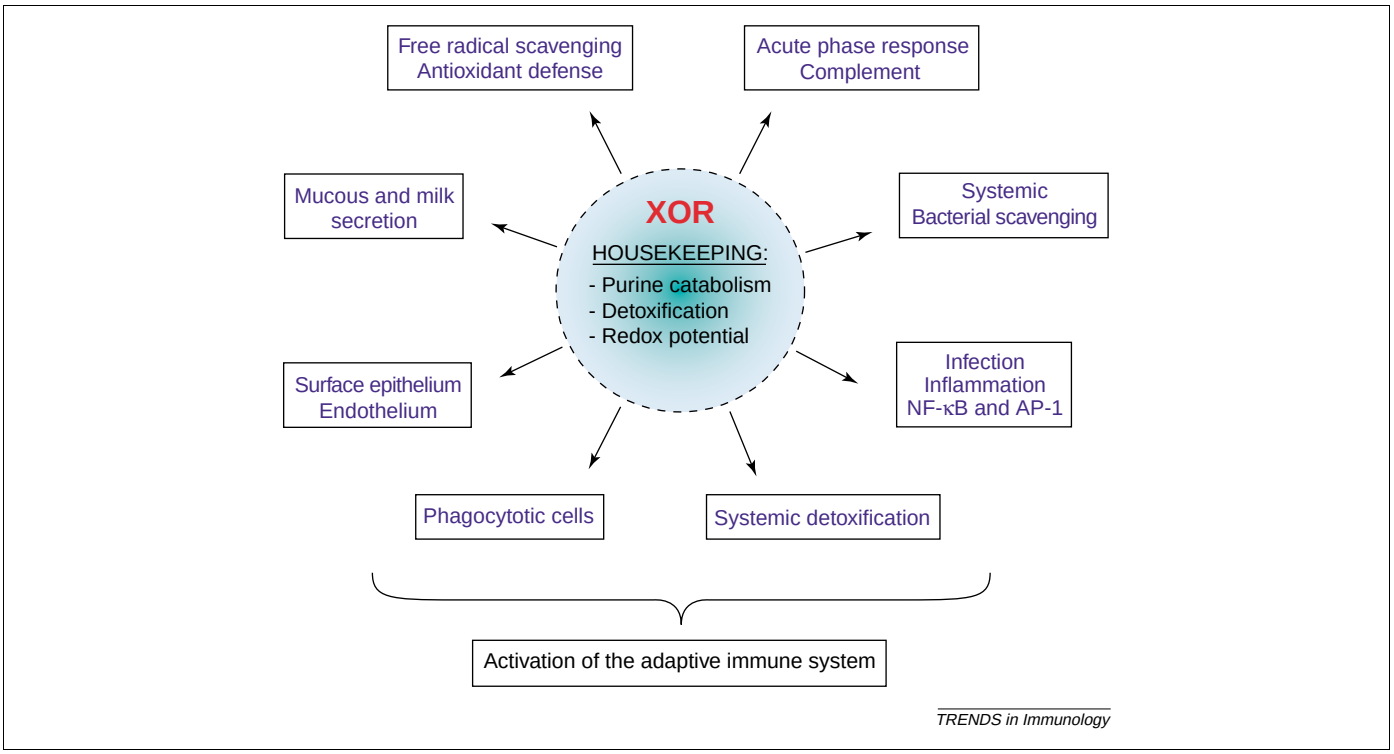

Fig. 2. XOR is an evolutionarily conserved housekeeping enzyme, important for purine catabolism, detoxification and the regulation of the cellular redox potential. Interestingly, the same protective functions are involved in multiple features of the innate immune system, suggesting that XOR is a central molecule in the evolution and function of this ancient defense system. Because innate immune responses generate many signals that co-stimulate the adaptive immune system it is possible that XOR also has a role in the crosstalk between innate and adaptive immunity. Abbreviations: AP-1, activator protein-1, XOR, xanthine oxidoreductase.

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