Hydrogen peroxide as second messenger in lymphocyte activation

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Oxidants such as H_2O_2 are connected to lymphocyte activation, but the molecular mechanisms behind this phenomenon are less clear. Here, I review data suggesting that by inhibiting protein tyrosine phosphatases, H_2O_2 plays an important role as a secondary messenger in the initiation and amplification of signaling at the antigen receptor. These findings explain why exposure of lymphocytes to H_2O_2 can mimic the effect of antigen. In addition, more recent data show that antigen receptors themselves are H_2O_2-generating enzymes and that the oxidative burst in macrophages seems to play a role not only in pathogen killing but also in the activation of these as well as neighboring cells. Thus, by controlling the activity of the negative regulatory phosphatases inside the cell, H_2O_2 can set and influence critical thresholds for lymphocyte activation.

Sometimes scientific progress is not based on a discovery or the generation of new data but on a change of viewpoint that allows one to see a set of already existing data in a new light. This is similar to an Escher painting, where a simple eye movement changes the interpretation of the picture. The study of the role of radical oxygen species (ROS) in the cell is such a case. ROS are a group of reactive oxygen species that include oxygen anions and radicals ($^{\cdot}O_2^-$ and OH·) or milder oxidants such as hydrogen peroxide (H_2O_2). There exists a huge body of data concerning the cell-damaging role of ROS[1]. The generation of ROS has been connected to stress responses, apoptosis, aging and death[2,3]. In recent years, however, the "bad reputation" of H_2O_2 and other ROS molecules has changed. These molecules are now being recognized as molecules of life that are essential to the proper development and proliferation of cells. It has been known for some time that low doses of H_2O_2 have mitogenic effects and can mimic the function of growth factors[4,5]. Only recently, however, has it become clear that these effects are not simply a reaction to an artificial exposure to ROS, but that upon stimulation by ligands, cells themselves produce H_2O_2 and use it as a second messenger for signal transduction and signal amplification[6,7]. Several excellent reviews about the function of H_2O_2 as a second messenger have appeared[8–13]. In addition, recent reviews have summarized the signaling functions of other members of the ROS molecular family, such as nitric oxide[14]. I will focus here on the role played by H_2O_2 in activation of the antigen receptor on lymphocytes. In addition, I will emphasize the concept that the initiation of antigen receptor signaling not only requires the activation of kinases but, more importantly, the inhibition of phosphatases and that the second messenger H_2O_2 is a critical element of such regulatory circuits.

H_2O_2 as second messenger

H_2O_2 shares several features with the well studied second messenger calcium[15]. It is a small molecule that can diffuse locally inside the cell. It is rapidly generated after an extracellular stimulus and can be easily removed by numerous mechanisms. Compared to other relatively short-lived ROS molecules—for example, the superoxide anion $^{\cdot}O_2^-$ (which has a half-life of 1 μs)—H_2O_2 is more stable (with a half-life of 1 ms), although its stability is influenced by the pH and the redox equilibrium inside the cell. More importantly, H_2O_2 is electrically neutral and is one of the few ROS molecules that diffuses freely through cellular membranes. Calcium acts *via* binding to calmodulin or other proteins with calcium-binding sites such as calcineurin or protein kinase C (PKC). H_2O_2 acts *via* the oxidation of proteins. Compared to the more aggressive ROS molecules—such as the hydroxyl radical OH·, which reacts with all molecules it encounters—H_2O_2 is a rather mild oxidant that primarily targets cysteine residues in diverse proteins.

Four oxidation states of cysteine can be generated: disulfide (-S –S), sulfenic acid (-SOH), sulfinic acid ($-SO_2H$) and sulfonic acid ($-SO_3H$). Generation of the latter two states requires strong oxidants such as pervanadate and their formation is irreversible under physiological conditions[16]. In contrast, H_2O_2 oxidizes the -SH group of cysteine to sulfenic acid, which is readily reduced to cysteine by various cellular reducing agents, including glutathione (GSH) and thioredoxin (Trx)[17]. However, the cysteine residue is only a good target for the oxidizing action of H_2O_2 if it is deprotonated and exists in the form of a cysteine thiolate anion (-S-). Most cysteine residues in proteins have a pK_a value of 8.5, and thus they do not exist as anions at physiological pH values. If, however, the cysteine residue is located in the vicinity of a positively charged amino acid, its pK_a value can be lowered to a pK_a below 5.0. Such a cysteine is deprotonated at physiological pH and becomes a target for the oxidizing action of H_2O_2. The fact that only certain proteins inside the cell carry an oxidizable cysteine at a critical position is the reason why such a small molecule like H_2O_2 can act as specific second messenger.

Several proteins can be oxidized and thus modified by H_2O_2. These redox-regulated proteins include transcription factors such as p53, Jun, Fos and the p50 subunit of NF-κB[18,19]. The oxidation of these proteins can either prevent (p53, Jun, Fos) or stimulate (p50) their transcriptional activity. A prominent—and for this review more relevant—group of redox-regulated proteins are the protein tyrosine phosphatases (PTPs)[20,21]. All PTPs contain, in their catalytic center, a

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reactive and redox-regulated cysteine in the vicinity of a positive charge with the sequence motif (HCxxGxxRS/T)[22,23]. This cysteine forms the thiol phosphate, an intermediate in the dephosphorylation reaction of PTPs. Oxidation of this cysteine residue by H_2O_2 renders the PTP completely inactive. As the oxidation of the PTP is reversible, PTPs exist in two alternate states: an active state with a reduced cysteine or an inactive state with an oxidized cysteine. Thus, like other intracellular proteins—for example, small G proteins (**Fig. 1a**)—PTPs are binary signaling elements **(Fig. 1b)**. The activation, or rather inactivation, of PTPs is controlled by extracellular signals and H_2O_2 plays a key role as a secondary messenger in this process.

How is H_2O_2 produced inside lymphocytes?

There are several sources of ROS production inside the cell. The reaction always starts with the transfer of an electron to molecular oxygen (O_2). This one-electron reduction results in production of the superoxide anion $\cdot O_2^-$ that, in contact with protons in the water, is rapidly converted to H_2O_2 and O_2 according to the formula $2\cdot O_2^- + 2H^+ \leftrightarrow H_2O_2 + O_2$. The conversion reaction occurs either spontaneously or is catalyzed by enzymes such as superoxide dismutase (SOD), which is found in the cytosol. During the conversion reaction, singlet oxygen is produced. This spin-activated form of molecular oxygen can be used to increase H_2O_2 production (see below). The electron transfers to O_2 can occur as leakage of the respiratory chain reaction in mitochondria, where some electrons can escape to generate $\cdot O_2^-$. Another source of ROS is the endoplasmic reticulum where $\cdot O_2^-$ is generated by NADPH cytochrome p450 reductase.

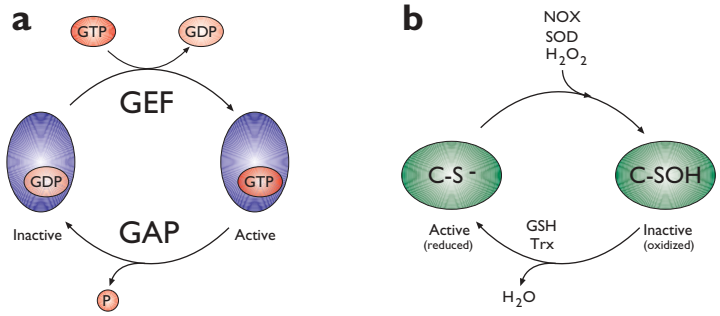

Figure 1. Regulation of binary intracellular signaling molecules. (**a**) Regulation of small G proteins. In resting cells, G proteins reside in an inactive GDP-bound state. Upon signaling they take up GTP, a process catalyzed by guanidine exchange factors (GEFs), and become active. The active stage is terminated by autocatalytic cleavage of the GTP phosphate, which can be enhanced by GTP-enhancing proteins (GAP). (**b**) Regulation of PTPs. In resting cells, PTPs are active and carry a deprotonated cysteine in their active center. Upon signaling, NADPH oxidases (NOXs) become active and produce, in conjunction with superoxide dismutase (SOD), H_2O_2 that oxidizes the cysteine to sulfenic acid (C-SOH) and renders PTP inactive. The reducing environment of the cytosol contains many redox regulators—such as glutathione (GSH) and thioredoxin (Trx)—which reduce sulfenic acid to cysteine, thereby reactivating the PTP.

The most relevant enzyme for the inducible production of ROS during signal transduction is the leukocyte NADPH oxidase (also known as respiratory burst oxidase)[24,25]. This plasma membrane–associated enzyme is best studied in phagocytes, but it also is found on other cells, for example, B lymphocytes. NADPH oxidase is a multicomponent enzyme that catalyzes the one-electron reduction of oxygen by NADPH. It comprises the membrane-bound flavocytochrome b558 (a heterodimer containing the subunits gp91^{phox} and p22^{phox}) and at least four cytosolic proteins: p47^{phox}, p67^{phox}, p40^{phox} (phox is short for phagocyte oxidase) and the small G protein Rac. During activation, the cytosolic proteins translocate to the plasma membrane and associate with cytochrome b558 to form the active superoxide-generating enzyme. The NADPH oxidase can be activated through different pathways. One of the activation routes involves the formation of GTP-bound Rac, which binds to p67^{phox} and recruits the p67^{phox}-p47^{phox} complex to cytochrome b558[26]. Another route for stimulation of the NADPH oxidase is the activation of PKC and other serine-threonine protein kinases[27]. These kinases phosphorylate p47^{phox}, which results in its activation and binding to cytochrome b558. The subunit p40^{phox} seems to have an inhibitory role by preventing formation of the active p67^{phox}-p47^{phox} complex[28]. Phosphorylation of p40^{phox} could release the inhibition and contribute to activation of the NADPH oxidase. The role played by PKCs in the activation of NADPH oxidase is demonstrated by the fact that B cells or macrophages treated with phorbol 12-myristate 13-acetate (PMA) display an oxidative burst[29,30].

During its catalytic reaction, the NADPH oxidase transfers electrons across the plasma membrane to extracellular oxygen. Thus the $\cdot O_2^-$ is generated in the extracellular space. As the $\cdot O_2^-$ anion is not membrane permeable, the products of NADPH oxidase activity can only modulate intracellular signaling pathways once they are converted into H_2O_2, which diffuses freely through cellular membranes. As already mentioned, the half-life of H_2O_2 is critically dependent on the redox equilibrium inside the cell. The cytosol is strongly reducing; this is due to redox regulators and cellular reductants such as GSH and Trx, which are abundantly expressed in all eukaryotic cells[17]. H_2O_2 oxidizes GSH into glutathione disulfide (GSSG), and enzymes such as the GSH reductase restore GSH. In addition, catalases are found at several cellular locations and these enzymes catalyze the conversion of H_2O_2 into water ($H_2O_2 + H_2O_2 \leftrightarrow 2H_2O + O_2$). Together these reductant molecules ensure that ROS products like H_2O_2 only have a short half-life inside the cell and thus can only act at a limited distance from their site of production.

Is there genetic evidence that the NADPH oxidase indeed plays a crucial role in intracellular signaling of lymphocytes? Several mutations with defects in components of the NADPH oxidase complexes have been found in human patients diagnosed with chronic granulomatous disease (CGD)[31]. Although these patients have a clear defect in macrophage and neutrophil function that causes this immune deficiency, they do not display the general block in lymphocyte development that one would expect from molecules playing a crucial role in lymphocyte activation. The same has been found in NADPH oxidase–deficient mice[32–34]. However, several homologs of gp91^{phox} have now been identified[35–38]. These proteins are expressed in many tissues and are called Nox1–Nox5. It is possible that homologs also exist for each of the cytosolic NADPH oxidase subunits. Thus, there surely is redundancy in the system, which would explain why the existing gp91^{phox} mutations do not result in a complete loss of H_2O_2 production[39]. In addition, as discussed above, the NADPH oxidase can be activated *via* alternative routes that may differ between macrophages and lymphocytes. This could result in differential sensitivity towards mutations of components of this multisubunit enzyme complex. The cytosolic subunits p47^{phox} and p67^{phox} are found in mature B cells but are not expressed in plasma cells, an expression pattern they share with other B cell antigen receptor (BCR) signaling components[30]. T lymphocytes produce H_2O_2 upon stimulation of their antigen receptor[40]. Yet, among

the known NADPH oxidase subunits, they only express p40^{phox}. Thus, either an alternative NADPH oxidase complex is expressed in T cells or these cells can generate ROS by an alternative route.

Immunoglobulins as H_2O_2-producing enzymes

Antibodies raised against molecules that mimic the intermediate of an enzymatic reaction have been used as a source of new enzymes[41,42]. In one of these tests, a H_2O_2-generating antibody was identified. Soon it was found, however, that this activity was shared by all tested immunoglobulins (Igs) and did not require a particular antigen-binding site[43,44]. In addition, a solubilized T cell antigen receptor (TCR) heterodimer (TCR$\alpha\beta$) also shows this enzymatic activity. As a substrate for this reaction, antibodies use singlet oxygen ($^1O_2$), an excited and more active oxygen state in which the oxygen atom has its two outer shell electrons spin-paired.

The electron donor for reduction of $^1O_2$ has been identified as water (H_2O). It is possible that this process involves formation of the intermediate H_2O_3 according to the formula $2H_2O + 2^1O_2 \leftrightarrow 2H_2O_3 \leftrightarrow 2H_2O_2 + O_2$. Several places for the catalytic center of this reaction are discussed[44]. The most likely one is the interface between the Ig heavy (H) and light (L) chain variable (V) domains, V_H and V_L, where several H_2O molecules are trapped in a hydrophobic pocket in a way that facilitates this reaction. However, the heavy chain constant (C) domains at the Fc-portion of Ig also have some H_2O_2-producing activity. Thus, it is possible that an H_2O_2-generating catalytic center is formed at the interface of the V and C domains of several members of the Ig superfamily.

One source of singlet oxygen is conversion of the NADPH oxidase–generated $^{\cdot}O_2^-$ anion to H_2O_2, as already mentioned ($2^{\cdot}O_2^- + 2H^+ \leftrightarrow H_2O_2 + {}^1O_2$). The colocalization of an antibody with an active NADPH oxidase can thus increase H_2O_2 production. It has been speculated that the H_2O_2-generating activity of antibody evolved in order to facilitate the killing of antibody-coated bacteria by ROS[45]. If this is true, then why does the TCR have the same reactivity? Another not mutually exclusive possibility is that the BCR, TCR and other Ig family members evolved the H_2O_2-generating activity to facilitate and amplify their signaling output, as will be discussed in detail below. A better understanding of the biological role of H_2O_2 production of antibodies requires the study of mutants that have lost their catalytic activity without being compromised in their assembly. Whether such mutants can be generated is not known at present.

How can H_2O_2 activate signaling?

Many receptors start to signal in a ligand-independent manner when cells are treated with either H_2O_2 or even stronger oxidants such as pervanadate. This indicates that H_2O_2 can mimic the function of the ligand. There are several possibilities as to how H_2O_2 could activate a receptor. H_2O_2 could directly oxidize receptor components and thus generate aggregation, cross-linking or conformational changes in these receptors that lead to their activation. Alternatively, H_2O_2 could activate intracellular protein tyrosine kinases (PTKs) involved in signal transduction from these receptors. The increased tyrosine-phosphorylation of PTK substrate proteins that occurs in H_2O_2-treated cells could be taken as evidence for this possibility. However, when PTKs are treated *in vitro* with H_2O_2, no increase in their kinase activity is detected[46]. The third possibility is that H_2O_2 inhibits PTP activity and thus allows receptors to signal in a ligand-independent fashion. According to simple signaling mathematics, the amount of phosphorylation inside the cell equals kinase activity minus phosphatase activity. Therefore, tyrosine phosphorylation of intracellular proteins can also be increased by the inhibition of PTPs, which are prominent targets of H_2O_2-mediated oxidation, as described above.

For a better understanding of the role played by H_2O_2 in receptor activation, a change in the view of how PTPs are involved in this process is required. By dephosphorylating PTK substrate proteins, PTPs are well known counter-elements of PTK activity. Therefore, PTPs are often seen as terminators of a signaling process initiated by receptor engagement and PTK activation. An alternative or extended view of PTP function is that PTPs are also directly involved in receptor activation. According to this view, receptors permanently signal *via* their kinase domains or associated PTKs unless they are prevented from doing so by PTPs functioning as a gatekeeper of receptor activation. Indeed, phosphatases are powerful negative regulators. In comparison to an active PTK, an active PTP has a 100–1000-times higher turnover rate and a race between the two enzymes is always won by the phosphatase. The reason for this is that substrate phosphorylation requires ATP and, thus, is a second-order reaction, whereas dephosphorylation is like a first-order reaction. Given this situation, one could wonder how kinases can phosphorylate their substrates at all in a cell that is full of phosphatases. Although the activity of many PTPs is regulated by intra- or intermolecular interactions (for example, dimerization), these enzymes remain more active than kinases[47,48]. Signal transduction should therefore not only involve PTK activation but, more importantly, PTP inhibition, and this is exactly what H_2O_2 can do. Elegant experiments have shown that engagement of the platelet-derived growth factor receptor by its ligand results in rapid H_2O_2 production and the transient oxidation and, thus, inhibition of the receptor-associated PTP SHP-2[49].

Role of H_2O_2 in BCR signaling

The interest of my group in the role of ROS in B cell activation dates back to the time when my students learned that by treating B cells with pervanadate instead of antigen they could generate stronger anti-phosphotyrosine immunoblots[46]. Several groups had, at that time, already described the effect of pervanadate on B cell activation[50,51]. Using an inducible system, we then could show that the effect of pervanadate required the expression but not the engagement of the BCR[52]. In pervanadate-treated cells, we did not detect BCR aggregation or capping, which suggested that this oxidant did not act directly on the BCR but rather on a signal element downstream from the receptor. What surprised me in these experiments was that pervanadate or H_2O_2, when used at the right concentration, induced phosphorylation of the same proteins that were phosphorylated upon antigen stimulation. Clearly, H_2O_2 treatment mimicked the exposure of B cells to antigen. These findings can now be explained by the essential role that H_2O_2 plays as a second messenger in antigen-dependent lymphocyte activation.

The BCR consists of the membrane-bound Ig molecule and the Ig$\alpha\beta$ heterodimer, which function as antigen-binding and signaling subunits, respectively[53,54]. The cytoplasmic tail of Igα and Igβ carries an immunoreceptor tyrosine–based activation motif (ITAM) characterized by a consensus sequence that includes two tyrosines that become phosphorylated upon BCR activation[55,56]. Three PTKs (Lyn, Syk and Btk) and one PTP (SHP-1) are involved in signal transduction from the BCR[57,58]. The generation of a doubly phosphorylated ITAM allows the two NH_2-terminal SH2 domains of Syk to bind to the BCR. This activates the kinase to phosphorylate neighboring ITAM sequences, which results in more Syk recruitment and activation and thus in the amplification of the BCR signal[59–61]. The cytosolic phosphatase SHP-1 is one of the PTPs counteracting Syk activity in B cells[62]. In the presence of

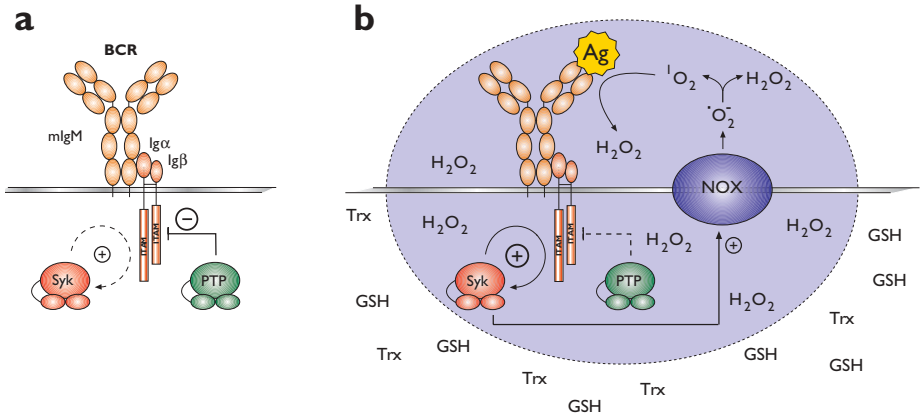

Figure 2. Model of the redox regulation of BCR signaling. (**a**) In the resting state of the BCR (a complex between membrane IgM (mIgM) and the Igαβ heterodimer), the signal-transducing kinase Syk cannot become activated at the ITAM, as any ITAM phosphorylation is prevented by dominant PTP activity. (**b**) Upon antigen (Ag) binding, the BCR is localized closed to a ROS-producing NADPH oxidase. The increased H_2O_2 production generates around the BCR an oxidizing environment or domain (dashed circle) that inhibits PTP, thus allowing Syk to become active. Signals through Syk and Lyn (data not shown) can further activate the NADPH oxidase, resulting in increased H_2O_2 production and spreading of the signal. During the conversion of $^{\bullet}O_2^-$ into H_2O_2, singlet oxygen ($^1O_2$) is produced that is reduced by the catalytic activity of Ig (here, mIgM) into H_2O_2. This process may help increase the oxidizing domain range around the BCR.

active SHP-1, the ITAM tyrosines are more rapidly dephosphorylated than they are phosphorylated by Syk. Therefore, no amplification of Syk activity can occur when the BCR is in a resting state (**Fig. 2a**). Upon exposure of B cells to H_2O_2 or upon stimulation of the H_2O_2-generating enzymes in B cells, the PTPs are inhibited and lose their negative regulatory power on the BCR. This results in rapid Syk activation and increased tyrosine phosphorylation (**Fig. 2b**).

What is the role of antigen in the process of BCR activation? In the reducing environment of the cytosol, H_2O_2 has only a short half-life and can act only close to its site of production. An important aspect of ligand-dependent BCR activation may be rapid translocation of the receptor to a source of H_2O_2 production or, *vice versa*, translocation of the source to the receptor. The membrane rafts may be one of the sites where the BCR and the oxidase could meet, but such colocalization has not yet been demonstrated[63,64]. Engagement of the BCR results in stimulation of H_2O_2 production[65] (and unpublished observations), which suggests that the NADPH oxidase and other ROS-producing enzymes are activated *via* BCR signaling. It is, thus, possible that the BCR and the NADPH oxidase are not only physically but are also functionally connected in a positive feedback loop. Signals *via* the BCR stimulate production of H_2O_2 inhibiting the PTPs around the BCR, thus increasing the BCR signal, which results in even more H_2O_2 production and so on. The result is rapid amplification of the BCR signal. According to this scheme, the activated BCR is surrounded by a cloud of H_2O_2. In this high oxidizing environment even unligated receptors could be activated and, like a neuronal action potential, spread the signal over the cell plasma membrane. Such ligand-independent signal spreading has indeed been described in cells expressing large amounts of the epidermal growth factor receptor[66].

The BCR could activate the NADPH oxidase *via* several alternative routes. One involves Src family kinases such as Lyn, which mediate phosphoinositide 3-kinase activation and the generation of phosphatidylinositol(3,4,5)trisphosphate that is bound by proteins *via* a pleckstrin-homology (PH) domain. One PH domain–containing protein is the GDP-GTP exchange factor Ras-GEF, which generates Rac-GTP and thus can activate the NADPH oxidase. An alternative route is phosphorylation of the adaptor protein SLP-65 (also known as BLNK or BASH) by Syk[67–69]. The phosphorylated adaptor recruits and activates Vav, a specific GDP-GTP exchange factor for Rac. The adaptor also activates phospholipase C-γ2 for the production of diacyglycerol, which stimulates PKC. The BCR-Syk-SLP-65–mediated production of Rac-GTP and activated PKC may act in concert to activate the NADPH oxidase[27].

In the first seconds after its engagement, the BCR cannot generate strong signals because it is still under the negative regulation of its associated PTPs. How, then, can the BCR signal reach the NADPH oxidase to start the signaling amplification process described above? Indeed, if the BCR is left alone with the PTPs, it has to fight an uphill battle and only rarely could reach the threshold set by the PTPs to prevent constitutive signaling. Here, as in other cases, cooperation between the adaptive and innate immune systems could play a role. Lipopolysaccharide (LPS) is a potent activator of NADPH oxidases, which suggests that Toll receptors can help the BCR start the H_2O_2-production and signal-amplification cycle[70,71]. On the other hand, the LPS response requires the BCR signaling machinery[72] and the second messenger H_2O_2 may be the molecule that communicates between these two receptor systems. BCR coreceptors could also act by increasing H_2O_2 production around the activated BCR.

H_2O_2 as an intercellular messenger?

After activation of the NADPH oxidase, H_2O_2 is first produced in the extracellular space and has to diffuse through the plasma membrane to act as a secondary messenger inside the cell. In the immune system, the activation of a lymphocyte often requires a close cellular contact between two cells forming a synapse. Such a synapse is formed between an antigen-presenting cell (APC) and a T cell as well as between B cells[73,74]. Due to the more oxidizing environment, H_2O_2 is likely to be more stable outside than inside the cell. H_2O_2 could therefore diffuse from an already activated cell with high H_2O_2 production to a cell that is being activated[75]. Thus, H_2O_2 may also function as a secondary messenger between cells. Neutrophils and macrophages are known to form the first line of defense against intruding pathogens. In addition, these cells produce large amounts of H_2O_2 during the oxidative burst reaction. So far, it is thought that the only role of the oxidative burst is to kill engulfed bacteria with the generated ROS. New data, however, show that proteases play a more important role than the ROS as antibacterial agents. Macrophages from mice deficient for the cysteine protease cathepsin G can no longer kill bacteria, although their oxidative burst is not impaired[76,77]. It is therefore possible that the oxidative burst is not primarily a killing device but rather a mechanism for activating the macrophage and neighboring lymphocytes. In the presence of H_2O_2, the dose response of antigen-specific activation of B lymphocytes is shifted to a lower antigen concentration. Thus, even B cells with a low-affinity BCR could be stimulated for antibody production if they are close to

a site of inflammation with many active H_2O_2-producing macrophages. Such low-affinity antibodies are indeed often found in the early phase of a bacterial infection[78]. Activated macrophages can function as APCs for T cell priming, and this process could be facilitated by H_2O_2 diffusing from the macrophage to the T cell during their close interaction phase. The full activation of macrophages requires their interaction with T helper (TH) cells. If the H_2O_2 production of macrophages is impaired, they may not be able to fully activate the TH cells and thus do not receive the signals required for their own maturation. In this respect, the immunodeficiency associated with CGD may not be primarily due to a defect in bacteria killing, but rather due to a defect in macrophage and lymphocyte activation. Defective TH cell activation has indeed been found in p47^{phox}-deficient mice[79].

Conclusion

The important role that H_2O_2 plays as an intracellular messenger is not well understood at present. The reason for this may be that H_2O_2 mostly acts only transiently and locally inside the cell and that its effects are hard to follow. Indeed, no method exists that can directly detect the H_2O_2-mediated oxidation of a redox-regulated protein in a way an anti-phosphotyrosine can detect a phosphorylated PTK substrate protein. However, at least an indirect method for detecting this modification has now been developed[49]. Due to the redundancy of the ROS-generating enzymes, the existing mouse mutants did not, in most cases, reveal the importance of H_2O_2 production for the activation and development of lymphocytes. On the other hand, this redundancy could be taken as evidence of how important the H_2O_2-producing system is for the life of a cell. Yet, at a recent signaling meeting, the word "H_2O_2" was not mentioned once, but this is likely to change in the near future. Well aware of the power of the H_2O_2-generating systems are our evolutionary companions the bacteria and viruses that love to fool our immune system. Why do so many bacteria produce catalase? Why do viruses often express proteins that either increase or reduce the H_2O_2 production in transfected cells[80]? Many diseases are characterized by an altered redox equilibrium[13]. Was the influenza A virus H1N1 of 1918 so deadly because it induces an oxidative burst in the infected lung cells that lead to an overreaction of the immune system[81,82]? The answers to these questions will not only help us better understand the diseases connected with these pathogens, but will also help us elucidate the diverse roles played by H_2O_2 in the activation process of lymphocytes.

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