Rapid burst of H_2O_2 by plant growth regulators increases intracellular Ca^{2+} amounts and modulates $CD4^+$ T cell activation

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Abbreviations: 2,4D, 2,4 Dichlorophenoxyacetic acid; $[Ca^{2+}]_i$, intracellular amounts of Ca^{2+}; DCFDA, 2', 7'– dichlorofluorescein diacetate; H_2O_2, hydrogen peroxide; HPA, 4-Hydroxyphenylacetic acid; IAA; Indoleacetic acid; I, Ionomycin; NAA, 1-Naphthaleneacetic acid; NAM, Naphthalene acetamide; PMA, phorbol 12-myristate 13-acetate; ROS, Reactive oxygen species; sol aCD3, soluble anti-CD3; SOS, strength of signal.

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ABSTRACT

The identification of small molecules that affect T cell activation is an important area of research. Three molecules that regulate plant growth and differentiation, but not their structurally similar analogs, were identified to enhance primary mouse $CD4^+$ T cell activation in conjunction with soluble anti-CD3 stimulation: Indoleacetic acid (natural plant auxin), 1-Napthaleneacetic acid (synthetic plant auxin) and 2,4-Dichlorophenoxyacetic acid (synthetic plant auxin and herbicide). These effects are distinct in comparison to Curcumin, the well known phenolic immunomodulator, which lowers T cell activation. An investigation into the mechanisms of action of the three plant growth regulators revealed a rapid induction of reactive oxygen species (ROS), mainly comprising H_2O_2. In addition, these three molecules synergize with soluble anti-CD3 signaling to enhance intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$, leading to greater T cell activation, e.g. induction of CD25 and IL-2. Enhanced production of TNFα and IFNγ by $CD4^+$ T cells is also observed upon plant growth regulator treatment with soluble anti-CD3. Interestingly, maximal IL-2 production and $CD4^+$ T cell cycle progression are observed upon activation with soluble anti-CD3 and phorbol 12-myristate 13-acetate (PMA), a phorbol ester. Additionally, stimulation with PMA and Ionomcyin (a Ca^{2+} ionophore), which activates T cells by circumventing the TCR, and plant growth

regulators also demonstrated the role of the strength of signal (SOS): T cell cycle progression is enhanced with gentle activation conditions but decreased with strong activation conditions. This study demonstrates the direct effects of three plant growth regulators on $CD4^+$ T cell activation and cycling.

1. Introduction

T cells act as global directors of the immune response. Consequently, lower numbers of T cells or deficiency in their function severely compromises immunity [1]. Not surprisingly, T cell activation has been the target for several therapeutic interventions. Most of these compounds, e.g. cyclosporine A, FK506, rapamycin etc, inhibit T cell responses and are important during organ transplantations, autoimmunity etc [2]. Also, compounds isolated from plants, e.g. curcumin and resveratrol, have been identified to modulate the immune response. Curcumin (diferulolylmethane, a principal component of turmeric) demonstrates profound anti-inflammatory effects, lowers inflammatory cytokines and immune cell proliferation, but enhances caspase activation and cell death [3]. It reduces autoimmune disease manifestations in animal models by modifying the ability of T cells to respond to cytokines, IL-12 and IFNβ [4]. Resveratrol (trans-3,5,4'-trihydroxystilbene), a polyphenolic compound (flavonoid) found in fruits like red grapes and cranberries, exhibits antioxidant and anti-inflammatory properties. It reduces mitogen- and antigen-induced T cell proliferation, allo-T cell responses and cytokine production *in vitro* [5]. Importantly, resveratrol decreases the clinical symptoms and inflammatory responses in experimental allergic encephalomyelitis (EAE)-induced mice by lowering the amounts of inflammatory cytokines and chemokines and enhancing death

of activated T cells [6]. Most studies have focused on inhibitors of T cell responses and there are fewer studies on small molecules that enhance T cell activation. However, nutritional supplements ranging from organic food extracts, plant components, vitamins and minerals may enhance immune responses and promote general health. For e.g., providing lipoic acid and acetyl carnitine reduces mitochondrial oxidative decay in lymphocytes [7]. Therefore, the identification of small molecules that modulate T cell activation is an active area of investigation.

$CD4^+$ T cells upon activation produce cytokines that increase T cell proliferation and modulate B cell and macrophage responses. $CD4^+$ T cell activation is driven by two distinct sets of signals: a primary signal mediated via the T cell receptor-CD3 complex and a second or costimulatory signal, which is vital for optimal T cell proliferation and survival [8]. The CD28/CTLA4 and CD80/CD86 family of receptors and ligands constitute the best known family of costimulatory molecules in T cell activation. An *in vitro* $CD4^+$ T cell activation system [9,10] was used to screen and identify small molecules that enhanced IL-2 production. This study describes three structurally distinct plant growth regulators, indoleacetic acid (IAA), 1-naphthaleneacetic acid (NAA) and 2, 4-dichlorophenoxyacetic acid (2,4D), to modulate $CD4^+$ T cell activation. The roles of plant growth regulator-induced intracellular intermediates, H_2O_2 and Ca^{2+}, in T cells are elucidated.

2. Materials and Methods

2.1 Mice

C57BL/6 mice, obtained from the Central Animal Facility, IISc, of either sex, aged 6-8 weeks, were used to isolate $CD4^+$ T cells.

2.2 Antibodies and chemicals

IAA, NAA, 2,4D, naphthylacetamide (NAM), hydroxyphenylacetic acid (HPA), catalase, Verapamil, EDTA and EGTA were obtained from Sigma Chemical Co., St. Louis, MO. Functional grade hamster control antibody, aCD3, aCD28 and antibodies used in flow cytometry, e.g. PE conjugated isotype control and aCD25, were obtained from eBioScience, San Diego, CA.

2.3 $CD4^+$ T cell isolation and activation

$CD4^+$ T cells were purified by complement-mediated depletion of $CD24^+$ B cells and $CD8^+$ T cells and panning on goat anti-mouse (Jackson ImmunoResearch, USA) coated

flasks. $CD4^+$ T cells were typically ~95% pure as measured by flow cytometry. Purified T cells were plated at ~6-7 x 10^4 cells/well in 96-well U-bottom plates (Becton Dickenson, Franklin Lakes, USA) in a final volume of 100 µl /well of RPMI 1640 supplemented with 5% FBS (Sigma). T cells were activated using 0.01 µg/ml soluble anti-CD3 (sol aCD3). Alternately, these cells were activated with pharmacological agents, 10 ng/ml of PMA and 0.1 or 0.5 µM of Ionomycin (I), unless otherwise mentioned. The isolation, culture and activation of $CD4^+$ T were accomplished, as described in detail in a previous publication [10].

2. 4 Cytokine assays

Supernatants from $CD4^+$ T cell assays were collected at 24 or 36 h after activation and cytokine levels were quantified using ELISA, according to the manufacturer's instructions (eBioscience).

2. 5 Flow cytometric analysis

For surface staining, ~2 x 10^5 cells were washed in cold HBSS (Sigma) containing 0.5% FBS and stained with appropriate pre-titrated PE conjugated antibodies. Flow cytometry was performed on FACScan (Becton Dickinson, San Jose, CA). Cell cycle analysis was performed using propidium iodide (Sigma), intracellular ROS was assessed using DCFDA (Calbiochem, San Diego, CA) and cytoplasmic free Ca^{2+} levels were measured with 1 µM Fluo-3 acetoxymethyl ester (Calbiochem). Flow cytometric analysis of cell surface expression, cell cycling, intracellular ROS and Ca^{2+} were performed as described in detail in a previous publication [10].

2. 6 Statistical analysis

Two – tailed paired Student's *t* test was performed to determine *p* values for individual experiments, as mentioned in figure legends.

3. Results

3.1 Plant growth regulators increase IL-2 production in CD4$^+$ T cells activated with aCD3

The ability of a select group of plant phenolic compounds (Fig. 1A) to enhance IL-2 production (Fig. 1B) and induce CD25, a T cell activation marker, (Fig. 1C) by CD4$^+$ T cells activated with sol aCD3 was studied. This screen identified the natural plant auxin, IAA and synthetic plant growth regulators, NAA and 2,4D, that enhanced IL-2 amounts and CD25 expression by ~ 2 fold (Fig. 1). Also, these effects were specific as no effect was observed upon addition of structurally similar compounds, NAM and HPA. The plant growth regulator-mediated increase in IL-2 was sensitive to cyclosporine, implicating the calcineurin pathway (Fig. 1D). Next, the effect of curcumin, the well known plant phenolic immunomodulator, was studied. As seen in Supplementary Fig. 1, curcumin decreased IL-2 production in sol aCD3 stimulated T cells. This experiment showed that plant growth regulators mediate effects distinct from curcumin.

3.2 Plant growth regulators induce $[Ca^{2+}]_i$ in a H_2O_2 dependent manner

IAA has been known to generate ROS and affect the proliferation of mammalian cells [11-13]. Therefore, the ability of these plant growth regulators to modulate ROS in T cells was studied. Incubation of NAA, 2,4D and IAA with cells led to a rapid oxidative burst in the absence and presence of aCD3 activation (Fig. 2A). The major species of ROS was identified to be H_2O_2, as addition of catalase decreased sol aCD3-induced IL-2 amounts (Fig. 2B).

One of the key factors that directly regulates IL-2 production is $[Ca^{2+}]_i$ [14]. The three plant growth regulators increased $[Ca^{2+}]_i$ only in cells activated with aCD3 (Fig. 2C). Also, the elevated $[Ca^{2+}]_i$ played a functional role as chelation with BAPTA-AM led to decreased IL-2 (Fig. 2D). Next, the kinetics of induction of ROS and $[Ca^{2+}]_i$ was studied. As observed in Fig. 3A, the induction of ROS, in the absence or presence of sol aCD3, was rapid (< 5 min) and sustained over 24 hr. On the other hand, $[Ca^{2+}]_i$ was induced only upon activation with sol aCD3 and peak amounts were observed ~12 hr (Fig. 3B). These results clearly demonstrate that induction of intracellular ROS by selected plant growth regulators was faster compared to the induction of $[Ca^{2+}]_i$.

As H_2O_2 and $[Ca^{2+}]_i$ were increased upon addition of selected plant growth regulators to $CD4^+$ T cells, the relationship of these two molecules was addressed. Upon addition of plant growth regulators, scavenging H_2O_2 with catalase greatly decreased ROS. Also, addition of superoxide and peroxynitrite scavengers did not affect plant growth regulator-induced ROS (data not shown). Interestingly, addition of catalase also lowered $[Ca^{2+}]_i$

(Fig. 4). On the other hand, the blockade of the influx of Ca^{2+}, using verapamil (L-type Ca^{2+} channel inhibitor), EDTA or EGTA (Ca^{2+}chelators) greatly reduced $[Ca^{2+}]_i$ but did not affect ROS amounts (Fig. 4). These results indicated that the plant growth regulators initially generated H_2O_2 which, together with aCD3 activation, increased $[Ca^{2+}]_i$ leading to IL-2 production.

3.3 Plant growth regulators induce cell cycling only in the presence of PMA

The addition of plant growth regulators to cells activated with sol aCD3 did not increase the percentage of cycling cells (Supplementary Fig. 2), which suggested that the IL-2 and CD25 induced by these compounds were insufficient to drive proliferation. Further increase in $[Ca^{2+}]_i$ by addition of I had no effect (data not shown). However, addition of PMA to these cultures greatly enhanced IL-2 (Fig. 5A) and CD25 (Supplementary Fig. 3A). Apart from IL-2, plant growth regulator treatment increased TNFα and IFNγ by ~ 2 fold whereas IL-4 was unchanged (Table 1). Notably, IL-2 (Fig. 5A) was more robustly increased (8 -12 fold), upon addition of PMA to cultures exposed to these three compounds and led to increased cell cycling (Fig. 5B). Also, plant growth regulator and PMA treated cells expressed higher amounts of proliferating cell nuclear antigen (PCNA), which is upregulated by rapidly dividing cells (Supplementary Fig. 3B). Most likely, in cells stimulated with sol aCD3, the plant growth regulator-enhanced $[Ca^{2+}]_i$ is insufficient for T cell cycling in the absence of optimum amounts of activated PKC. These results are summarized in a model (Fig. 6).

3. 4 Effect of plant growth regulators on cell cycling is dependent on the SOS

To test the roles of plant growth regulators in a TCR-independent model of activation, cells were treated with an optimal concentration of PMA (10 ng/ml) and different concentrations of I. The addition of these compounds increased IL-2 in cells activated with PMA and 0.1 μM I (Fig. 7A). Stimulation of cells with just PMA and 0.5 μM I greatly enhanced IL-2 production (strong signal) and the plant growth regulator-induced IL-2 effect was not observed. Also, the three compounds increased T cell cycling in cells activated with PMA + 0.1 μM I (Fig. 7B). However, addition of these three compounds in PMA + 0.5 μM I activated cells decreased cell cycling (Fig. 7B). Therefore, these three plant growth regulators enhanced IL-2 and proliferation in mild activation conditions (sol aCD3, PMA + 0.1 μM I) but decreased proliferation in cells activated with stronger activation conditions (PMA + 0.5 μM I).

4. Discussion

This study identified three structurally distinct small molecules, belonging to the plant growth regulator family to influence $CD4^+$ T cell activation. These molecules act only in concert with a primary signal (e.g. sol aCD3) to increase IL-2 (Fig. 1B) and the surface expression of T cell activation marker, CD25 (Fig. 1C). T cells used in this study were predominantly $CD4^+CD25^-$ (unactivated, considered functionally naive) which expressed CD25 and CD69 upon activation. $Foxp3^+$ regulatory T cells tend to express significant levels of CD25 irrespective of activation [15]. Based on the low amounts of activation markers, CD25 and CD69, it is unlikely that the T cells used in the study were regulatory T cells. Also, FoxP3 expression is lowered upon activation of $CD4^+CD25^-$ T cells [16]. Notably, while the effects occured directly on $CD4^+$ T cells, no functional effects with respect to IL-2 secretion or proliferation were observed by the selected compounds on their own (data not shown). It is also unlikely that IAA, NAA and 2,4D are signaling via

the costimulatory CD28 pathway as demonstrated by the effects of cyclosporine A (Fig. 1D). Furthermore, the inclusion of PMA greatly enhanced IL-2 amounts (Fig. 5A) and cell cycling (Fig. 5B) in $CD4^+$ T cells activated with IAA, NAA and 2,4D. These compounds alone could not enhance cell cycling in cells activated with sol aCD3 (Supplementary Fig. 2) suggesting induction of a state of partial activation. Also, addition of PMA increased CD25 expression (Supplementary Fig. 3A) and levels of cell cycle protein PCNA (Supplementary Fig. 3B) which could explain the dramatic increment in cell cycling. As previously shown, the activation by sol aCD3 is gentle (low SOS) and significant proliferation requires greater help (e.g. PMA) or costimulation by aCD28 [9,10]. It is important to point out that the ability of NAA, 2,4D and IAA to regulate T cell activation was specific as structurally similar compounds, NAM and 4HPA, did not show any effect.

To gain better mechanistic insights, the modulation of two intracellular signaling molecules by plant growth regulators was studied: ROS and Ca^{2+}. Here, we show that IAA, NAA and 2,4D alone, but not NAM and HPA, in a dose-dependent manner (Fig. 2A) rapidly enhanced intracellular ROS in the presence and absence of sol aCD3 activation (Fig. 3A). Studies with catalase identified plant growth regulator-induced H_2O_2 to play the major role in this response (Figs. 2B & 4). Previous studies have demonstrated that IAA after oxidation in the presence of horseradish peroxidase [11-13] generates intermediates that are cytotoxic to tumor cells. The molecule responsible for IAA mediated cytotoxicity was shown to be H_2O_2 released during the oxidation of IAA by peroxidase [13]. Given that there were no obvious structural similarities between IAA,

NAA and 2.4D, the ability to enhance intracellular ROS was the phenotype that clearly distinguished these compounds from the control compounds in the absence of stimulation with aCD3. Further studies are required to understand the processes by which ROS is rapidly generated in T cells.

TCR triggering has been shown to produce ROS which plays functional roles during T cell proliferation, differentiation, cytolysis and cell death [17-19]. In fact, glutathione depletion lowers IL-12 production and maturation of dendritic cells which affects the generation of the Th1 response [20]. Also, mice lacking NADPH oxidase develop severe collagen-induced arthritis, a T cell dependent inflammatory disease [21]. In fact, the oxidative amount of thiols on the T cell surface membrane has been shown to be important in modulating arthritis in rats [22]. Importantly, treatment of rats with phytol, which increases oxidative burst *in vivo* lowers autoimmune inflammatory responses in a rat model of arthritis [23]. Together these studies demonstrate the key role of ROS in modulating *in vitro* and *in vivo* T cell responses. Therefore, observations that the three plant growth modulators generate rapid ROS and modulate T cell responses are important. A notable aspect is the high amount of compounds required for the induction of IL-2 by sol aCD3 stimulated $CD4^+$ T cells. Importantly, dose response experiments clearly demonstrated that lower amounts of the compounds (100 μM) were sufficient to upregulate ROS and $[Ca^{2+}]_i$. These three plant growth regulators significantly enhanced $[Ca^{2+}]_i$ only in the presence sol aCD3 activation and significant IL-2 production by these compounds required higher amounts (e.g. 500-1000 μM). It is possible that at low concentrations of the compounds, the threshold levels of Ca^{2+} are insufficient to increase

IL-2. In fact, H_2O_2 is known to increase IL-2 production in Jurkat T cells [24]. Therefore, it is not surprising that NAA, 2,4D and HPA increase IL-2 levels and activation by increasing H_2O_2 in $CD4^+$ T cells activated with a weak signal. Studies with inhibitors clearly demonstrated that the rapid increase in H_2O_2 was responsible for an influx of extracellular Ca^{2+} which increased $[Ca^{2+}]_i$ (Fig. 4), which plays important roles in T cell activation [14]. This aspect is important as low amounts of ROS are generated after TCR stimulation. However, excess ROS causes oxidative stress, resulting in lower proliferation and increased cell death [17,18].

These data have been incorporated in a model to explain plant growth regulator-induced T cell activation with sol aCD3 (Fig. 6). Most likely, IAA, NAA and 2,4D strengthen the TCR mediated calcineurin sensitive signal transduction pathway. Therefore, stimulation of the PKC pathway by PMA greatly enhances T cell cycling by these compounds in conjunction with sol aCD3 stimulation. Interestingly, studies with a TCR-independent system of activation using PMA and I, clearly showed the role of the SOS in determining the functional outcome of the plant growth regulator response (Fig. 7B). The compounds enhanced IL-2 amounts and cell cycling during stimulation with PMA and low amounts of I (0.01 - 0.1 μM). On the other hand, IL-2 amounts were not affected but cell cycling was reduced upon activation with PMA and higher amounts of I (0.5 μM). Thus, in two T cell activation conditions (sol aCD3 and PMA + I), IAA, NAA and 2,4D enhanced $CD4^+$ T cell cycle progression. Also, NAA, 2,4D and IAA increase IL-2 levels and activation by increasing H_2O_2 in $CD4^+$ T cells activated with a mild signal. However, in cells activated with a strong signal which already posses high amounts of ROS [10], the

enhanced H_2O_2 amounts suppresses cell growth. In fact, cell cycle analysis clearly demonstrated that these compounds significantly modulate cell cycling (Fig. 7B) compared to cell death (Supplementary Fig. 4). Most likely, the effects of these three plant growth regulators on T cell activation and proliferation is greater compared to their effects on T cell survival.

It is important to discuss the implications of our data on the possible *in vivo* roles of IAA, NAA and 2,4D. In general, IAA is non-toxic to adult mice; however, administration of 500-1000 mg/kg during the mid-gestation period causes microencephaly in the fetus [25]. IAA administration (500 mg/kg) lowers the expression of antioxidant enzymes in the liver and is protective in a model of diethylenitrosoamine-induced hepatocarcinogenesis in mice [26]. The activation of IAA using peroxidase or UV medium wavelength light results in the production of cytotoxic oxidation products that is effectively during tumor therapy [27]. Previous studies on the roles of 2,4D in animal systems have largely been confined to toxicity studies, and have not really shed much light on physiological pathways that might be invoked. Animal studies have shown that casual exposure to 2,4D does not lead to toxic or teratogenic effects, though very high doses can damage liver and kidneys, and irritate mucous membranes [28]. Orally fed NAA as well as 2,4D have been shown to be non-toxic to rats at 5 mg/kg body weight [29]. Animal experiments with oral feeding of N-butyl esters of 2,4D did not show any toxicity. However, at high amounts, enhanced T and B cell proliferation in response to mitogens was observed [30]. On the other hand, another study reported that 2,4D, at high doses, suppresses T cell proliferation in response to ConA [31]. These studies clearly demonstrate that plant

growth regulators do affect immune response in vivo although further studies are required to understand the differences in results obtained.

Lowered immune function is observed during late stages of cancer, microbial infections (e.g. AIDS), old age etc. However, there is a paucity of information on the identity of compounds and mechanisms by which T cell activation can be enhanced. These studies are important as such compounds regulate T helper cell differentiation [32], cytotoxicity [33] and apoptosis [34], all of which have implications in inflammatory responses, enhancing the efficacy of vaccines and in the treatment diseases, e.g. cancer [35] and HIV infection [36]. This is the first study to show the ability of IAA, 24D and NAA to rapidly induce ROS and increase Ca^{2+} in $CD4^+$ T cells *in vitro*. Importantly, the roles of these selected plant growth regulators in modulation of *in vitro* $CD4^+$ T cell proliferation depending on SOS were demonstrated. Further studies will be useful to determine potential applications of these plant growth regulators in regulating T cell immune responses during disease conditions.

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Figure Legends

Fig. 1. Plant growth regulators increase IL-2 production in activated $CD4^+$ T cells in a Cyclosporine A-sensitive manner. (A) Structures of the small molecule plant growth regulators used in this study. (B) $CD4^+$ T cells were activated with sol aCD3 in the absence or presence of different concentrations of the indicated compounds. IL-2 levels were measured in culture supernatants after 24 h by ELISA. (C) $CD4^+$ T cells were activated with sol aCD3, with or without 500 μM of indicated compounds, and CD25 expression was studied by flow cytometry after 12 and 36 h of activation. The fold differences in MFI were calculated with respect to the MFI of cells stained with isotype control antibody the value for which was considered unity. All data shown is mean +/- SE from three independent experiments. Indicated *p* values, ** $p < 0.05$, * $p < 0.1$, are for NAA, 2,4D and IAA. (D) $CD4^+$ T cells were activated with sol aCD3 in the presence or absence of 500 μM of NAA, NAM, 2,4D, HPA and IAA. Different concentrations of cyclosporine A were added and IL-2 levels were measured in culture supernatants after 24 h. Data shown is mean +/- SE from three independent experiments.

Fig. 2. Plant growth regulators increase intracellular ROS and $[Ca^{2+}]_i$ amounts. $CD4^+$ T cells, either unactivated (- aCD3) or activated with sol anti-CD3 (+ aCD3) in the presence or absence of different concentrations of indicated compounds were cultured for 1 h and relative ROS (A) and $[Ca^{2+}]_i$ (C) levels were measured. Fold differences in MFI were plotted either with respect to unactivated (- aCD3) or sol aCD3 (+ aCD3) activated cells whose MFI was taken as unity. Different concentrations of catalase (B) and BAPTA-AM (D) were added to cells activated with sol aCD3 +/- 500 μM of indicated compounds and IL-2 levels were measured after 24 h. All data shown is mean +/- SE from three independent experiments. Indicated P values, ** $p < 0.05$, are for NAA, 2,4D and IAA compared to NAM and HPA.

Fig. 3. Plant growth regulators rapidly increase intracellular ROS. $CD4^+$ T cells, either unactivated (- aCD3) or activated with sol anti-CD3 (+ aCD3) in the presence or absence of indicated compounds were cultured for indicated time periods and relative ROS (A) and $[Ca^{2+}]_i$ (B) amounts were measured. All data shown is mean +/- SE from three independent experiments. Indicated p values, *** $p < 0.001$ and * $p < 0.05$, are for NAA, IAA and 2,4-D compared to NAM or HPA respectively.

Fig. 4. Plant growth regulator-enhanced ROS enhances $[Ca^{2+}]_i$. Different concentrations of catalase, Verapamil, EDTA and EGTA were added to sol aCD3 activated cells to which 500 μM of the compounds had either been added as indicated and relative ROS and $[Ca^{2+}]_i$ levels were measured after 1 h. Fold differences in MFI were calculated with

respect to untreated cells which were considered as unity. Data shown is mean +/- SE from three independent experiments.

Fig. 5. Activation with sol aCD3, together with PMA, enhances cell cycling in plant growth regulator treated cells. (A) T cells activated with sol aCD3 were treated with 500 μM of indicated compounds in the presence of different concentrations of PMA. IL-2 amounts in culture supernatants were measured by ELISA after 24 h. (B) Cell cycle analysis was performed after 48 h and fold differences in percentage of S/G2M cells were calculated with respect to cells activated with aCD3 alone. Data shown is mean +/- SE from three independent experiments. Indicated P values, * $p < 0.1$, are for NAA, 2,4D and IAA compared to control compounds.

Fig. 6. Proposed model for plant growth regulator action in $CD4^+$ T cells. Plant growth regulators cause a rapid oxidative burst which, in the presence of aCD3 signaling, leads to increase in $[Ca^{2+}]_i$. In combination with PMA, this increase in $[Ca^{2+}]_i$ greatly increases IL-2 amounts and cell cycling.

Fig. 7. Plant growth regulators modulate T cell cycling upon activation with PMA and I. (A) $CD4^+$ T cells were activated with PMA (10 ng/ml) and different concentrations of I, together with 500 μM of indicated compounds. IL-2 amounts were measured after 24 h. (B) $CD4^+$ T cell were activated with 10 ng/ml PMA + 0.1 or 0.5 μM I in the absence or presence of 500 μM of compounds and cell cycle analysis was performed after 48 h. Fold differences in percentage of S/G_2M cells under different activation conditions were

normalized to cells activated with PMA + 0.1 μM I the value for which was taken as unity. Data shown is mean +/- SE from three independent experiments. Indicated *p* values, ** $p < 0.05$, * $p < 0.5$, are for NAA, 2,4D and IAA compared to NAM and HPA.