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Granulocytes as models for human protein maker identification following nicotine exposure

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Abstract

Nicotinic acetylcholine receptors (nAChRs) are pentameric cation channels expressed in the mammalian CNS, in the peripheral nervous system, and in skeletal muscle. Neuronal-type nAChRs are also found in several non-neuronal cell types, including leukocytes. Granulocytes are a subtype of leukocytes that include basophils, eosinophils, and neutrophils. Granulocytes, also known as polymorphonuclear leukocytes, are characterized by their ability to produce, store, and release compounds from intracellular granules. Granulocytes are the most abundant type of leukocyte circulating in the peripheral blood. Granulocyte abundance, nAChR expression, and nAChR upregulation following chronic nicotine administration makes granulocytes interesting models for identifying protein markers of nicotine exposure. Nicotinic receptor subunits and several non-nAChR proteins have been identified as protein markers of granulocyte nicotine exposure. We review methods to isolate granulocytes from human tissue, summarize present data about the expression of nAChRs in the three granulocyte cell types (basophils, eosinophils, and neutrophils), describe current knowledge of the effects of nicotine exposure on human granulocyte protein expression, and highlight areas of interest for future investigation.

Keywords

granulocytes; nAChRs; nicotine

Nicotinic acetylcholine receptors (nAChRs) are pentameric cation channels that are expressed in the mammalian CNS, in the peripheral nervous system, and at neuromuscular junctions (Millar and Gotti 2009). Eleven neuronal nAChR subunits have been identified in mammals (α2–7, α9–10, β2–4) (Albuquerque *et al.* 2009). nAChRs are endogenously stimulated by acetylcholine, as well as by nicotine, the primary addictive component in cigarettes (Palmer *et al.* 2005).

Nicotine activates nAChRs when administered acutely, and elicits additional effects in the CNS when used chronically. It was first shown in 1985 that during chronic exposure, some nAChRs subtypes are increased in number, as well as in sensitivity to nicotine (Marks *et al.* 1985; Schwartz and Kellar 1985). This phenomenon, termed ‘upregulation’, has been observed in both rodent and primate models of nAChR expression (Breese *et al.* 1997; Peng

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et al. 1997; Perry *et al.* 1999; Marks *et al.* 2011; Alsharari *et al.* 2015). The degree of receptor upregulation following chronic nicotine exposure displays selectivity with regard to brain region and to nAChR subtype. Upregulation takes place posttranslationally, because nicotine has been shown to serve as a pharmacological chaperone for nascent nAChRs (Henderson and Lester 2015). Several groups are pursing the hypothesis that nAChR upregulation is both necessary and sufficient to explain the early stages of nicotine dependence (days to weeks). Therefore, nAChR upregulation has the potential to become a mechanistically revealing biomarker for chronic nicotine exposure. The study of neuronal nAChRs in human brain tissue is not practical for clinical use, leading to the desire for more readily available tissue in which to identify markers of nicotine exposure.

In addition to being expressed in the mammalian CNS, 'neuronal' nAChRs are also found in peripheral blood cells, including leukocytes. This raises the possibility that biomarkers for chronic nicotine exposure could be found in blood. Granulocytes, also known as polymorphonuclear leukocytes (PMNs), are distinguished from other white blood cells by the presence of intracellular granules as well as by variable nuclear morphologies. There are three types of granulocytes: basophils, eosinophils, and neutrophils (Geering *et al.* 2013).

Each type of granulocyte has a distinct set of functions in the immune system. The granules of basophils and eosinophils produce cytokines, small proteins that are involved in the coordination of immune and allergic responses. Eosinophils also produce reactive oxygen species in response to immune challenges (Blanchet *et al.* 2007; Geering *et al.* 2013). Neutrophils attack bacteria and other microorganisms by phagocytosis and/or by the release of toxic effector molecules contained in intracellular granules (Boxio *et al.* 2004). The majority of circulating granulocytes are neutrophils, representing 50–70% of total leukocytes in blood (Rodak and Carr 2016). As a result of the abundance of neutrophils in PMN/granulocyte populations, the terms PMN, granulocyte, and neutrophil are often used interchangeably in the literature. To avoid confusion, this review uses the term granulocyte to refer to total granulocytes.

The expression of nAChRs has also been observed in other leukocytes, including lymphocytes, macrophages, and mast cells (Fujii and Kawashima 2001; Fujii *et al.* 2008; Kageyama-Yahara *et al.* 2008; Kindt *et al.* 2008; Yamamoto *et al.* 2014). However, granulocytes display several attributes that make them advantageous models for identifying protein markers of human nicotine exposure. As mentioned above, granulocytes, in particular the highly abundant neutrophil, vastly outnumber other leukocytes such as lymphocytes in peripheral blood (Benhammou *et al.* 2000; Rodak and Carr 2016). Millions of human granulocytes can be isolated from peripheral blood using typical blood draw volumes (Lebargy *et al.* 1996). In addition, while neutrophil number is increased in smokers versus non-smokers, mononuclear leukocytes (e.g. lymphocytes) do not increase in number (van Eeden and Hogg 2000; Sorensen *et al.* 2004).

Identification of nicotine exposure protein markers in humans is of interest because such markers could serve several goals including: the assessment of compliance with smoking cessation therapy; the comparison of effects of different tobacco products; the comparison of nicotine use in different populations of smokers; and the correlation of nicotine use with

varying degrees of addiction. These markers could also aid in understanding responses to different active molecules and flavorings in cigarettes (e.g. menthol).

Granulocyte isolation

Murine granulocytes can be isolated from several tissues including bone marrow, kidney, spleen, peripheral blood, and peritoneal fluid (Williams *et al.* 1977; Boxio *et al.* 2004; Coquery *et al.* 2012; Swamydas *et al.* 2015; Safronova *et al.* 2016). Isolating neutrophils from bone marrow or the peritoneal cavity after challenge with a pro-inflammatory compound is a common method used in mouse studies (Devi *et al.* 1995; Boxio *et al.* 2004; Tanaka *et al.* 2004; Safronova *et al.* 2016). It should be stressed that the source tissue of the neutrophil, the stage of cell development, and the source species may influence the observed responses to nicotine administration. Several signaling pathways have been reported to differ between the granulocytes of mice and humans (Geering *et al.* 2013).

While many informative granulocyte studies have been conducted in rodents, this review primarily focuses on work using granulocytes isolated from human peripheral blood to maximize biological and medical relevance of protein markers for smoking. Cabanis *et al.* (1994) reviews several methods for isolating granulocytes from human peripheral blood, with varying degrees of purity. All of these methods involve gradient or differential centrifugation to separate granulocytes from other leukocytes and a hemolysis step to remove erythrocytes. Many of the granulocyte studies included in this review utilize a variation of these methods (Lebargy *et al.* 1996; Benhammou *et al.* 2000; Iho *et al.* 2003; Cormier *et al.* 2004). Isolating murine neutrophils from peripheral blood in a similar fashion is possible, but is more difficult because of limited blood volume (Devi *et al.* 1995; Swamydas *et al.* 2015).

As a result of their lower cell number in human peripheral blood, the isolation of basophils and eosinophils is more complicated than the isolation of neutrophils. The isolation of basophils and eosinophils from total granulocyte preparations can be achieved by positive or negative selection of cell populations using basophil, eosinophil, and neutrophil specific protein markers. To isolate eosinophils, total granulocytes are isolated as described above and incubated with beads conjugated with anti-CD16 antibodies. Targeting CD16, a protein expressed on neutrophils, removes neutrophils from the total granulocyte preparation (Blanchet *et al.* 2007; Watson *et al.* 2014). To isolate both basophils and eosinophils, cell populations can be selected for being CD45 and FcεR1-positive cells (basophils), and for being CD45-positive, CD16-negative cells (eosinophils) (Watson *et al.* 2014).

Expression of nAChRs in granulocytes

Neuronal nAChRs and nAChR subunits serve as biomarkers for chronic nicotine exposure in the CNS of both rodents and humans (Breese *et al.* 1997; Perry *et al.* 1999; Marks *et al.* 2011; Alsharari *et al.* 2015). This leads to the possibility that nAChRs in granulocytes could serve more conveniently as a biomarker of nicotine exposure in humans. To evaluate this possibility, it is important to know which nAChR subtypes are expressed in granulocytes, and if they upregulate in response to nicotine exposure.

The expression of nAChR in granulocytes has been investigated in both rodents and humans. Because the data for nAChR expression in human granulocytes are less complete, several rodent studies are discussed that provide preliminary information to guide investigations of human granulocyte nAChR expression.

For a comprehensive understanding of nAChRs in granulocytes, the expression of nAChRs can be investigated on the transcript (mRNA), the protein (nAChR subunit), and intact receptor levels. RT-PCR analysis of granulocytes isolated from human peripheral blood identified the mRNA transcripts for nAChR subunits α3, α4, α7, β2 and β4 (Benhammou *et al.* 2000). Shown in Table 1, transcripts for all neuronal nicotinic receptor subunits have been identified in rodent granulocytes (Safronova *et al.* 2016).

Expression of α3, α4, α7, β2, and β4 nAChR subunits in human granulocytes has been confirmed using western blotting (Benhammou *et al.* 2000). Querying the assembly of subunits into dimers using co-immunoprecipitation further detected robust α3β4 dimer formation and lower levels of α4β2 formation (Benhammou *et al.* 2000).

Radioligand binding assays are a technique used to investigate ligand interactions with assembled intact nAChRs. High-affinity [^{3}H]-nicotine binding was observed on granulocytes isolated from non-smokers (Hoss *et al.* 1986; Lebargy *et al.* 1996; Benhammou *et al.* 2000). A second class of very high-affinity nicotine binding sites was only observed in granulocytes isolated from smokers (Lebargy *et al.* 1996; Benhammou *et al.* 2000). Similarly, [^{3}H]-epibatidine binding sites have been reported in human granulocytes isolated from smokers (Cormier *et al.* 2004). Epibatidine binding has been characterized in the mammalian CNS to represent primarily binding to the α4β2 subtype receptor, though epibatidine may also bind to α3β2, α3β4, and α7-nAChRs with different kinetics (Whiteaker *et al.* 2000). These radioligand binding studies were all normalized to either purified granulocyte protein or granulocyte cell number, accounting for any changes in total granulocyte count in response to chronic nicotine. While demonstrated levels of nicotine binding in granulocyte preparations are lower than that observed in the mouse whole brain and the human cortex, they are comparable to levels observed in several human and rodent brain studies investigating quantitative changes in nAChRs in response to nicotine exposure (Marks & Collins 1982; Breese *et al.* 1997). The α7-nAChR subtype was detected in both murine and human granulocytes as determined by binding of α-bungarotoxin (α-bgtx), which is generally considered a selective antagonist for α7-nAChRs (Table 1) (Cormier *et al.* 2004; Safronova *et al.* 2016). Data generated by Safronova *et al.* (2016) provide insight into the nAChR subtypes that are represented in murine granulocytes by assaying the sensitivity of granulocyte substrate adhesion to α-cobratoxin, α-conotoxin MII and α-conotoxin PnIA exposure. Like α-bgtx, α-cobratoxin is an antagonist of α7-nAChRs, while α-conotoxin MII is an antagonist of α3β2 and α3α6β2-nAChRs and α-conotoxin PnIA is an antagonist of α3β2-nAChRs. Their work suggests possible expression of α3β2 and α3α6β2-nAChRs in murine granulocytes, though a comparably elegant study has not yet been performed using human cells. The presence of α3β2-nAChRs in human, non-smoker granulocytes has been suggested previously based on the observation that the relatively high amount of nicotine required to elicit interleukin 8 (IL-8) induction surpassed the nicotine concentration required to activate α4β2-nAChRs (Iho *et al.* 2003). Taken together, the expression of α3,

α4, α7, β2, and β4 nAChR subunits has been confirmed in human granulocytes. In addition, experimental data reveal that these expressed receptor subunits assemble into intact α7-nAChRs capable of binding known nAChR antagonists, as well as into presumptive α4β2, α3β2, α3α6β2, and/or α3β4-nAChRs (Table 1).

The study of nAChR expression has been more limited in basophils and eosinophils than in the total granulocyte population. Basophils isolated from allergic patients express α4 subunit containing nAChRs (α4* nAChRs) and α7-nAChRs, confirmed by flow cytometry data. Immunoreactivity was demonstrated using an antibody reactive with α1, α3, and α5, though more work is required to learn which of the three subunits are expressed in human basophils (Watson *et al.* 2014). Expression of α3, α4 and α7 subunit transcripts and protein in human eosinophils has been reported using a combination of RT-PCR, ELISA, and immunohistochemistry (Blanchet *et al.* 2007).

Expression of α7-nAChRs in human leukocytes may be confounded by the presence of CHRFAM7A, a partially duplicated gene for the α7 nAChR subunit (reviewed by Costantini *et al.* 2015a). The protein product of CHRFAM7A, dupα7, has been identified in lymphocytes, but not yet in granulocytes (Villiger *et al.* 2002; Benfante *et al.* 2011). However, it is possible that dupα7 is expressed in granulocytes but at currently undetectable levels. The expression of this gene, and its regulatory effect on α7-nAChRs, is still being explored. Importantly, CHRFAM7A is expressed only in humans; thus its possible involvement with α7-nAChR functions or with nAChR-independent functions, in response to nicotine exposure will not be represented in rodent studies (de Lucas-Cerrillo *et al.* 2011; Costantini *et al.* 2015a,b; Sinkus *et al.* 2015).

nAChRs as peripheral protein markers of nicotine usage

Reported studies suggest that nAChRs themselves can be peripheral protein markers for human nicotine exposure. An increase in high-affinity [^{3}H]-nicotine binding has been shown in granulocytes isolated from smokers versus non-smokers (Table 2) (Lebargy *et al.* 1996; Benhammou *et al.* 2000). In concordance with [^{3}H]-nicotine binding, Cormier *et al.* (2004) also noted an increase in the number of [^{3}H]-epibatidine binding sites on the granulocytes isolated from smokers (Table 2). Granulocytes do express α3, α4, and β2 nAChR subunits, all of which can form nAChRs that upregulate in response to chronic nicotine in the CNS (Henderson and Lester 2015). These nicotinic receptor subtypes and their constituent subunits expressed in granulocytes are prime candidates to be protein markers of human nicotine exposure.

Of particular interest is the potential to use β2 subunit levels as a biomarker for nicotine exposure. In general, nAChRs containing β2 subunits (β2* nAChRs) upregulate in the mammalian CNS in response to chronic nicotine administration (Marks *et al.* 2011; Alsharari *et al.* 2015). In the human CNS, β2* nAChRs have been shown to up-regulate in some brain regions of smokers compared to non-smokers (Staley *et al.* 2006; Brody *et al.* 2013). Expression of β2 transcripts and subunits have both been confirmed in human granulocytes (Benhammou *et al.* 2000). In addition to α4β2-nAChRs, several studies suggest that α3β2-nAChRs are expressed, though co-immunoprecipitation investigations for

α3 and β2 have not yet been reported in human granulocytes. Evidence of cytisine-resistant epibatidine binding and α-conotoxin MII affinity, both of which are attributed to α3β2-nAChRs, has not yet been reported in granulocytes (Marks *et al.* 2000; Whiteaker *et al.* 2000). Together, these studies suggest that β2* nAChRs are possibly associated with the observed nicotine and epibatidine binding. Additional work is required to confirm which specific β2* nAChRs subtypes are expressed in granulocytes and which subtypes may upregulate in response to nicotine.

While α7-nAChR expression has been identified in granulocytes, α-bgtx binding does not increase in cells isolated from smokers compared to non-smokers (Cormier *et al.* 2004). It is possible that α7 nAChR subunits upregulate in granulocytes without assembling into receptors, but this possibility has not yet been investigated.

[^{3}H]-nicotine binding levels observed in granulocytes isolated from ex-smokers have been observed to remain increased for up to 8 months after smoking cessation (Lebargy *et al.* 1996). In the same study it was also observed that nicotine binding in granulocytes of ex-smokers finally decreased toward non-smoker levels after smoking cessation for > 12 months. This differs from observations in the human CNS, where the duration of nicotine-induced nAChR upregulation subsides in postmortem brain tissue from ex-smokers after 2 months (Breese *et al.* 1997).

Circulating, mature granulocytes live for hours to days in the blood, suggesting that the persistent increase in [^{3}H]-nicotine binding levels is not principally occurring as a result of granulocyte exposure to nicotine in the blood (Simon and Kim 2010; Kolaczkowska and Kubes 2013; Tak *et al.* 2013). Several studies have demonstrated that nicotine has an effect on granulocyte precursor cells. Granulocyte precursors are known as myeloblasts, which in turn arise from hematopoietic stem cells in the bone marrow (Summers *et al.* 2010). A model of human myeloblasts is the promyeloblast cell line HL-60, which can differentiate into granulocyte-like cells (Villiger *et al.* 2002; Xu *et al.* 2008). HL-60 cells have demonstrated α7 and dupα7 expression as well as altered non-nAChR protein expression and altered function in response to nicotine treatment (Armstrong *et al.* 1996; Villiger *et al.* 2002; Xu *et al.* 2008). The expression of α1–7, α9–10, and β1–β4 subunit transcripts, as well as the expression of α3, α4, α7, and β4 subunits, has been demonstrated in human stem cell models of hematopoietic tissue development (Serobyan *et al.* 2007). In addition, murine hematopoietic stem cells have been demonstrated to express α7 subunit transcripts and bind α-bungarotoxin, suggesting the presence of intact α7-nAChRs (Chang *et al.* 2010). The prolonged upregulation of nAChRs observed in mature circulating granulocytes may therefore be not only from exposure to nicotine during their short life span in circulating blood, but also from exposure during one or more earlier stages in granulocyte development as myeloblasts or hematopoietic stem cells. Hematopoietic stem cells have life spans greater than 10 months, which is consistent with the 8 month increase in [^{3}H]-nicotine post-smoking cessation in ex-smokers (Sieburg *et al.* 2011).

Non-nAChR peripheral protein markers

Non-nAChR proteins may also be useful as markers for nicotine exposure in granulocytes. In addition to nAChR upregulation, several non-nAChR proteins have been reported to have altered expression levels in granulocytes exposed to nicotine (Table 3). Depending on the technique used, changes in protein expression can be observed either on the surface of the cell or in the total cell. A key distinction among the studies reporting changes in non-nAChR protein expression is the nicotine exposure model used. The majority of studies use one of two models. The first is to isolate granulocytes from non-smoking subjects and to expose them to nicotine. The second strategy is to isolate and compare granulocytes from smokers with non-smokers. Table 3 highlights non-nAChR peripheral protein markers identified using these two experimental approaches.

The identification of non-nAChR protein markers of nicotine exposure in human granulocytes is potentially important since nicotine use affects immune function, as comprehensively reviewed elsewhere (Palmer *et al.* 2005; Kawashima *et al.* 2012; Olofsson *et al.* 2012; Martelli *et al.* 2014). Neutrophils, the principal granulocyte population, act through a repertoire of reactive oxygen and nitrogen-generating processes collectively known as respiratory burst (reviewed by Palmer *et al.* 2005). Microorganisms are killed by these reactive species. Proteins involved with cellular recruitment from the bone marrow, or neutrophil adhesion, have also been associated with neutrophil immune function (Iho *et al.* 2003). Several of the protein markers highlighted in Table 3 reported to have altered expression in response to nicotine are involved with these processes and may be associated with the changes seen in immune function following nicotine use.

Potential non-nAChR protein markers of nicotine exposure identified in human granulocytes include: IL-8, L-selectin (CD62L), lymphocyte function-associated antigen 1 (LFA-1), macrophage-1 antigen (MAC-1), matrix metallopeptidase 9 (MMP-9), myeloperoxidase (MPO), nuclear factor κ-light-chain-enhancer of activated B cells inhibitor alpha (IκBα), and nuclear factor κ-light-chain-enhancer of activated B cells inhibitor beta (IκBβ).

Expression of CD62L is a prime example of responses that can differ among experimental nicotine exposure models. Three groups have reported investigations of the expression of CD62L in response to nicotine, using two different methodologies. Speer *et al.* (2002) observed a decrease in CD62L levels, using granulocytes isolated from non-smokers, and exposed *ex vivo* to nicotine. van Eeden and Hogg (2000) however noted an increase in CD62L in smokers when comparing granulocytes isolated from smokers versus non-smokers. Ryder *et al.* (1998) similarly compared CD62L in granulocytes from smokers versus non-smokers, though with a smaller cohort than van Eeden and Hogg, and observed a decrease in smokers (Table 3). Because granulocytes from the van Eeden & Hogg and Ryder *et al.*, studies were exposed chronically *in vivo* to nicotine and to other cigarette smoke components, observed changes in protein expression compared to non-smokers may be especially informative.

Similarly, various effects have also been reported for LFA-1 and MAC-1 protein expression in granulocytes depending on the experimental model of nicotine exposure. LFA-1 and

MAC-1 are both integrins involved in cell adhesion. One study reported no observable differences between LFA-1 protein expression in granulocytes of smokers versus non-smokers (Ryder *et al.* 1998). A second study using non-smoker granulocytes that were exposed *ex vivo* to nicotine demonstrated a decrease in LFA-1 protein expression (Speer *et al.* 2002). The effect of *ex vivo* nicotine on MAC-1 protein expression in granulocytes of non-smokers was investigated by two groups, both of which observed a decrease in expression in response to nicotine (Speer *et al.* 2002; Vukelic *et al.* 2013). Comparing MAC-1 protein expression in smokers versus non-smokers, however, did not demonstrate a change in expression following chronic cigarette smoke exposure (van Eeden and Hogg 2000).

IL-8 administration increases the number of granulocytes exiting the bone marrow into the circulation; and IL-8 protein expression increased when granulocytes from non-smokers were exposed to nicotine *ex vivo* (Iho *et al.* 2003). IκBα and IκBβ are inhibitors of NF-κB, a transcription factor that regulates the IL-8 gene. Both inhibitors show decreased protein expression in response to nicotine exposure *ex vivo* in granulocytes isolated from non-smokers (Iho *et al.* 2003). Comparing smokers and non-smokers, van Eeden and Hogg 2000 observed an increase in MPO expression on the surface of granulocytes using flow cytometry. MPO is an enzyme involved in production of the reactive species that are involved with neutrophil respiratory burst (van Eeden and Hogg 2000). These four non-nAChR proteins all exhibit roles in neutrophil function and may serve as protein markers of nicotine exposure.

Several non-nAChR proteins have been highlighted as possible protein markers of nicotine exposure in granulocytes. Differences among the reported effects of nicotine or tobacco exposure on expression of several of these identified proteins may be attributed to differences between the models of nicotine exposure used, differences in cohort characteristics and size, and/or differences in analytical technique. We note that most studies used analytical techniques that investigated cell surface and not total cellular expression. Further analysis is required to validate these protein markers for different models of nicotine exposure. Additional work is also required to expand the current list of possible non-nAChR protein markers.

Several studies using isolated basophils and eosinophils have also highlighted changes in non-nAChR protein expression in response to non-nicotine cholinergic drugs that may also be markers of nicotine use. Dimethylphenylpiperazinium and di-ethyl-phenylhomopiperazinium are two experimental cholinergic agonists with anti-inflammatory properties (Watson *et al.* 2014). Activation of nAChRs following dimethylphenylpiperazinium exposure decreases MMP-9 protein expression in eosinophils stimulated with 5-oxo-eicosatetraenoic acid. Matrix metallopeptidases such as MMP-9 are involved with extracellular matrix remodeling and have been implicated with chronic obstructive pulmonary disease (COPD) as well as asthma (Blanchet *et al.* 2007; Grzela *et al.* 2016). CD203c, a marker for basophil activation, exhibited decreased levels in basophils isolated from peripheral blood of subjects with allergies (Table 3). Di-ethyl-phenylhomopiperazinium acts on both nicotinic and muscarinic acetylcholine receptors; the decrease in CD203c expression was ablated with hexamethonium, a non-selective nicotinic

antagonist, and with α-bgtx. This nicotinic antagonist-sensitive decrease suggests that a decrease in allergen-induced basophil activation is due in part due to nAChR activation (Watson *et al.* 2014). In summary, despite the currently limited information about protein changes in human basophils and eosinophils in response to nicotine exposure, protein markers do change when other nicotinic agonists stimulate nAChRs in human basophils and eosinophils.

Tools for identifying granulocyte biomarkers

The study of nicotine/tobacco usage requires the identification of protein biomarkers that can objectively facilitate the monitoring of nicotine exposure. Since humans are genetically diverse and demonstrate diverse nicotine/tobacco use behaviors, identifying multiple biomarkers may be useful to monitor nicotine/tobacco exposure for different populations, different tobacco products and different delivery systems. Ideally, to establish the utility of biomarkers, potential markers should be identified using multiple methods in well characterized large sample sizes. Changes in nicotine/tobacco exposed groups could be correlated or associated with certain changes observed only in specific subpopulations of individuals. These subpopulations may include for example individuals with challenged immune systems, or individuals intentionally or unintentionally (i.e. secondhand smoke) exposed to nicotine.

By pursuing unbiased methods, such as protein identification using mass spectrometry, we can better understand what total changes in protein expression are occurring in response to nicotine and the method of administration (e.g. cigarettes vs. electronic cigarettes). There are also non-nicotine substances in tobacco products that may be biologically active that could effect protein expression (Rabinoff *et al.* 2007; Ahijevych and Garrett 2010; Talhout *et al.* 2011; Wickham 2015; van de Nobelen *et al.* 2016). Like nicotine, the levels of these non-nicotine substances could also vary by tobacco product and by the method of delivery. Therefore, studying one biomarker may not be equally useful for all populations and for all tobacco products and methods of delivery. While many studies have investigated changes in expression of various proteins in response to nicotine or tobacco, a global approach has not yet been pursued in human granulocytes. Immunoassays (e.g. western blots, flow cytometry, etc.) are common methods of assessing proteomic changes. While the number of proteins investigated by these methods is relatively limited since they require specific antibodies, these methods highlight the possible tools that could be used to compare the effects of tobacco use across different populations once biomarkers are established.

The predominant method of quantifying general cholinergic expression of human nAChRs in granulocytes has been [^{3}H]-nicotine or [^{3}H]-epibatidine binding on the cell surface or in homogenates. A further refinement, using affinity purification of homogenates with nAChR antibodies prior to epibatidine binding has been reported in studies of the CNS (Marks *et al.* 2011; Fasoli *et al.* 2016). This combination of affinity purification with radioligand quantitation could provide valuable information on changes in nAChR levels in response to nicotine. Additionally, while qualitative immunoblotting for human granulocyte nAChR expression has been reported in the literature, quantitative studies following nicotine stimulation have not been reported. Immunoblots have previously been used to quantify

changes in nAChR subunit expression in other models such as the mammalian CNS and would provide valuable information about how nAChR subunits and other proteins are affected by chronic nicotine in granulocytes (Parker *et al.* 2004; Hussmann *et al.* 2014; Alsharari *et al.* 2015; Fasoli *et al.* 2016; Pistillo *et al.* 2016).

Several studies have demonstrated the capability of mass spectrometry to identify general protein changes in response to chronic nicotine in a variety of neuronal and, non-neuronal tissues of both mice and humans (Nordman *et al.* 2014; Paulo *et al.* 2015; McClure-Begley *et al.* 2016). The unique strength of mass spectrometry is the unbiased ability to identify novel changes in protein expression in various populations.

Value may be added by the investigation of biomarkers in basophils, eosinophils, and neutrophils in addition to total granulocytes. Affinity purification for one or more of the available basophil, eosinophil, and neutrophil selection markers, coupled with immunoblotting or mass spectrometry, could result in the identification of important biomarkers that may be overlooked in total granulocyte preparations.

Summary and future

Proteins that are upregulated or downregulated in response to nicotine may be useful as markers of nicotine/tobacco exposure. Granulocytes offer several advantages as potential models in which to identify protein markers. They are abundant in peripheral blood, obtainable from reasonable blood draw volumes, and endogenously express nAChRs. The expression of nAChRs may serve as protein markers of nicotine usage since they have been shown to upregulate in response to nicotine exposure. Nicotinic receptors and nicotinic receptor subunits therefore are of interest as protein markers of nicotine use. Epibatidine and nicotine binding increase in granulocytes of smokers versus non-smokers, suggesting nAChRs that contain α3, α4, and/or β2 subunits increase in smokers. Non-nAChR peripheral protein markers in human granulocytes may also prove to be useful nicotine exposure markers. The reported variation in the effects of nicotine exposure on expression of several of these potential protein markers highlights the necessity for further investigation using consistent models of nicotine exposure. Ideally, to reflect the true clinical effect of nicotine and cigarette smoke, comparisons of protein markers should be made in granulocytes isolated from smokers versus non-smokers.

It is also possible that nAChRs have functions independent of their role as ion channels. These additional functions may be stimulated naturally by acetylcholine, or pathologically by chronic exposure to nicotine. This possibility highlights the potential involvement of proteins physically interacting with nAChRs (Iho *et al.* 2003; Cloez-Tayarani and Changeux 2007; Paulo *et al.* 2009; Lester *et al.* 2012; Nordman and Kabbani 2012; Nordman *et al.* 2014). Identification of non-nAChR protein expression changes using mass spectrometry, either by querying total proteomic changes in response to chronic nicotine or specifically focusing on proteins interacting with nAChRs, may lead to the identification of protein markers in smokers and non-smokers. Mass spectrometry has already been used to investigate nAChR involvement in immune function in a recent investigation of $CD4^+$ T

lymphocytes (Nordman *et al.* 2014). Applied to granulocytes, this approach could become a powerful tool to identify cohorts of protein markers following nicotine use.

The identification of additional protein markers unique to different populations of nicotine users may yield therapeutically beneficial information. Some of the human studies reviewed here incorporate an assessment of nicotine dependence or addiction, such as the Fagerström test for nicotine dependence (Heatherton *et al.* 1991). Additional studies evaluating changes in protein expression may allow for correlation between protein markers and assessments of dependence/addiction.

Nicotine usage, commonly through cigarette smoking, is an important medical and public health issue. The landscape of nicotine delivery products is changing and tools to evaluate, to identify potentially harmful effects and to regulate these new products are required. In addition to changes in the expression of intact nAChRs and nAChR subunits, several non-nAChR proteins have been shown to have altered protein expression in granulocytes of smokers compared with non-smokers. These points highlight granulocytes as an ideal model for the identification and use of peripheral protein markers of nicotine usage. Additional study of granulocytes may enable the identification of reliable protein markers that can contribute to the understanding of the effects of chronic nicotine usage in humans.

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Abbreviations used

5-oxo-ETE	5-oxo-eicosatetraenoic acid
ACh	acetylcholine
ASM-024	di-ethyl-phenylhomopiperazinium
CD62L	L-selectin
DMPP	dimethylphenylpiperazinium
IL-8	interleukin 8
IκBα	nuclear factor κ-light-chain-enhancer of activated B cells inhibitor alpha
IκBβ	nuclear factor κ-light-chain-enhancer of activated B cells inhibitor beta
LFA-1	lymphocyte function associated antigen 1
MAC-1	macrophage-1 antigen
MMP-9	matrix metallopeptidase 9
MPO	myeloperoxidase
nAChR	nicotinic acetylcholine receptor

NFκB	nuclear factor κ-light-chain-enhancer of activated B cells
PMNs	polymorphonuclear leukocytes
α-bgtx	α-bungarotoxin

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Table 1

Expression of nAChRs in human and murine granulocytes

Granulocyte preparation	Species	mRNA transcripts	Protein	Receptor	Reference
Basophil	H	N.I.	α1/α3/α5, α4, α7	α1/α3/α5*, α4*, α7*	Watson *et al.* 2014
Eosinophil	H	α3, α4, α7	α3, α4, α7	N.I.	Blanchet *et al.* 2007
Total granulocyte	H	N.I.	α3, α4, α7, β2, β4	Nic, α3β4, α4β2	Benhammou *et al.* 2000
Total granulocyte	H	N.I.	N.I.	EB, α-bgtx	Cormier *et al.* 2004
Total granulocyte	H	N.I.	N.I.	Nic	Hoss *et al.* 1986
Total granulocyte	H	N.I.	N.I.	α3β2	Iho *et al.* 2003
Total granulocyte	H	N.I.	N.I.	Nic	Lebargy *et al.* 1996
Total granulocyte	H	α7	α7	N.I.	Villiger *et al.* 2002
Total granulocyte	M	α2–7, α9, β2–4	N.I.	α3β2, α3α6β2, α-bgtx	Safronova *et al.* 2016

H, Human; M, Murine; N.I., Not investigated.

Confirmation of nAChR transcripts (mRNA), subunits (protein), and/or assembled receptors distinguished by granulocyte type and species. Granulocyte preparation indicates whether total granulocytes were isolated or if basophils and eosinophils were investigated specifically. Studies using binding of ligands nicotine (Nic), epibatidine (EB), or α-bungarotoxin (α-bgtx) are noted.

Table 2

Changes in nicotinic ligand binding in granulocytes of smokers compared to non-smokers

[^{3}H]-Nicotine	[^{3}H]-Epibatidine	[^{125}I]-α-bungarotoxin	Reference
Increase			Lebargy *et al.* 1996, Benhammou *et al.* 2000
	Increase	(N.S.)	Cormier *et al.* 2004

N.S., not significant.

Effects of smoking on nicotine, epibatidine, and α-bgtx binding. Nicotine and epibatidine primarily bind to α3*, α4*, β2*, and β4* nAChRs while α-bgtx binds to α7-nAChRs selectively. Comparing granulocytes of smokers to non-smokers, increases in binding were observed for both nicotine and epibatidine, but no increase in binding was observed for α-bgtx.

Table 3

Potential non-nAChR peripheral protein markers of human nicotine exposure

Protein	Granulocyte preparation type	Granulocyte comparison	Effect on protein expression	Protein expression	Technique	Reference
Ectonucleotide pyrophosphatase/ phosphodiesterase family member 3 (CD203c)	Basophil	Allergic subject cells (+/−) ASM-024	Decrease with ASM-024	Surface	FC	(Watson *et al.* 2014)
Interleukin 8 (IL-8)	Total granulocyte	Non-smoker (+/−) nicotine	Increase with nicotine	Total	RT-PCR	(Iho *et al.* 2003)
L-selectin (CD62L)	Total granulocyte	Smoker versus non-smoker	Decrease in smokers	Surface	FC	(Ryder *et al.* 1998)
L-selectin (CD62L)	Total granulocyte	Non-smoker (+/−) nicotine	Decrease with nicotine	Surface	IHC	(Speer *et al.* 2002)
L-selectin (CD62L)	Total granulocyte	Smoker versus non-smoker	Increase in smokers	Surface	FC	(van Eeden and Hogg 2000)
Lymphocyte function-associated antigen 1 (LFA-1, CD11a, CD18)	Total granulocyte	Smoker versus non-smoker	No difference	Surface	FC	(Ryder *et al.* 1998)
Lymphocyte function-associated antigen 1 (LFA-1, CD11a, CD18)	Total granulocyte	Non-smoker (+/−) nicotine	Decrease with nicotine	Surface	IHC	(Speer *et al.* 2002)
Macrophage-1 antigen (MAC-1, CD11b)	Total granulocyte	Non-smoker (+/−) nicotine	Decrease with nicotine	Surface	IHC	(Speer *et al.* 2002)
Macrophage-1 antigen (MAC-1, CD11b)	Total granulocyte	Smoker versus non-smoker	No difference	Surface	FC	(van Eeden and Hogg 2000)
Macrophage-1 antigen (MAC-1, CD11b)	Total granulocyte	Non-smoker (+/−) nicotine	Decrease with nicotine	Surface	FC	(Vukelic *et al.* 2013)
Matrix metallopeptidase 9 (MPP-9)	Eosinophil	Asthmatic subject cells + 5-oxo-ETE (+/−) DMPP	Decrease with 5-OXO + DMPP	Total	CoIP/WB	(Blanchet *et al.* 2007)
Myeloperoxidase (MPO)	Total granulocyte	Smoker versus non-smoker	Increase in smokers	Surface	FC	(van Eeden and Hogg 2000)
NF-κ-B inhibitor α (IκBα)	Total granulocyte	Non-smoker (+/−) nicotine	Decrease with nicotine	Total	WB	(Iho *et al.* 2003)
NF-κ-B inhibitor β (IκBβ)	Total granulocyte	Non-smoker (+/−) nicotine	Decrease with nicotine	Total	WB	(Iho *et al.* 2003)

ASM-024, di-ethyl-phenylhomopiperazinium; DMPP, dimethylphenylpiperazinium; 5-oxo-ETE, 5-oxo-eicosatetraenoic acid; nAChR, nicotinic acetylcholine receptor; CoIP/WB: co-immunoprecipitation coupled with western blot analysis FC, flow cytometry; IHC, immunohistochemistry; RT-PCR, reverse transcription polymerase change reaction; WB, western blot.

The comparison made in the study, the demonstrated relationship in relative expression is shown, as well as the utilized technique and the location of expression changes. Total granulocyte preparations represent primarily neutrophils whereas basophils and eosinophils were investigated specifically.