

Immunology and genetics of tumour necrosis factor in allergic contact dermatitis

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Summary

During the sensitization phase of allergic contact dermatitis, the proinflammatory cytokine tumour necrosis factor (TNF) plays an important role by promoting epidermal Langerhans cell migration to draining lymph nodes. It also plays a role during the elicitation phase. The TNF gene (*TNF*) is located within the major histocompatibility complex region. Many single-nucleotide variants exist in the promoter region of *TNF*, and these may either increase or decrease mRNA transcription and therefore lead to higher or lower levels of TNF. The most extensively studied single-nucleotide variant of *TNF* is a base pair substitution in the promoter region at location –308 relative to the transcription start site (rs1800629, *TNF* –308G>A), which is believed to increase transcription and lead to higher TNF levels. The role of TNF in allergic contact dermatitis and the functionality of *TNF* –308G>A are reviewed in this article. The association between genetic variants and disease can be studied in a case–control design. Only a few case–control studies investigating the association between *TNF* –308G>A and allergic contact dermatitis have been published, with contradictory results. These are reviewed critically, and suggestions for future case–control studies on this topic are made.

Key words: allergic contact dermatitis; genetics; immunology; *TNF* –308G>A; tumour necrosis factor.

Allergic contact dermatitis (ACD) is a delayed-type hypersensitivity reaction caused by exposure of the skin to allergens (1). It starts with a sensitization phase, in which a person gains the potential to develop a cutaneous allergic reaction to a certain allergen, i.e., acquires contact allergy. Repeated exposure to this allergen (or a cross-reacting allergen) in a sensitized person causes inflammation of the skin. This is called the elicitation phase. The level of exposure, determined by dose and time of exposure, is the most important exogenous factor determining whether an individual becomes sensitized. However, a certain amount of interindividual variability

exists in the susceptibility to develop ACD independently from exposure (2), some examples being sex, ethnicity, and age (3, 4). There may, however, also be a genetic basis for this variability in susceptibility, as has been suggested by family and twin studies (5–8). Knowledge of the genetic factors that lead to an increased susceptibility to develop ACD could help in identifying individuals at risk. Genetic factors for the development of ACD that have been investigated so far have previously been reviewed by Schnuch et al. and Friedmann et al. (9, 10)

A large part of genetic research is focused on ‘candidate genes’, that is, genes that encode specific proteins relevant to the disease pathophysiology (11). One such important protein is tumour necrosis factor (TNF) (sometimes still referred to as tumour necrosis factor alpha, or TNF- α) (12). TNF is a proinflammatory cytokine with important roles in many inflammatory diseases, for example in psoriasis and rheumatoid arthritis (12). The role of TNF in ACD has been extensively investigated. The most

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important function seems to be to promote migration of the antigen-presenting Langerhans cells (LCs) to draining lymph nodes during the sensitization phase (13–15). TNF also has an important role during the elicitation phase of ACD (16). Genetic variants in the promoter region of the TNF gene (*TNF*) can lead to either an increase or a decrease in transcription of *TNF*, and can therefore influence TNF production (17). They can therefore theoretically lead to a higher or lower susceptibility to develop contact allergy and subsequent ACD. The role of these genetic variants of *TNF* in ACD has so far been studied in a few different clinical studies (18–24).

This review aims to elucidate the role of TNF in ACD, look at the different variants in the promoter region of *TNF* and their biological relevance, take a critical look at the clinical studies performed on *TNF* variants in ACD, and give suggestions for future research on variants in *TNF*.

The Role of TNF in Contact Hypersensitivity

TNF is an important player in the development of contact hypersensitivity by inducing LC migration

One of the essential steps in the sensitization phase of ACD is considered to be the migration of hapten-carrying LCs from the epidermis to draining lymph nodes. LCs belong to the dendritic cell (DC) family, and are the main antigen-presenting cell residents in the epidermis. When they arrive in the paracortical area of the lymph node, LCs present the hapten to naive T lymphocytes. The T lymphocytes then differentiate into antigen-specific type 1 T helper (Th) cells/type 1 cytotoxic T (Tc) cells and Th17/Tc17 cells, and proliferate. More recent evidence suggests that dermal DCs are vital for hapten presentation to T cells and their differentiation into effector T cells, with LCs having a more tolerogenic role (discussed in more detail below) (25, 26).

LC migration is initiated by altered expression of its surface adhesion molecules. This maturation process is induced by cytokines, among them TNF, released in the epidermis after penetration of a hapten into the epidermis. Experimental studies have shown the important role of TNF: intradermal injection of TNF induced DC accumulation in regional lymph nodes, and injection with anti-TNF inhibited LC migration (13–15). The following sections will examine the role of TNF in ACD more deeply, and discuss how TNF production in the epidermis is caused by exposure to haptens, how TNF induces LC migration, and the role of TNF in the elicitation phase.

TNF production and secretion occurs after activation of the innate immune system by the hapten

TNF secretion in the epidermis occurs early in the sensitization phase. Its secretion is a result of the activation of the innate immune system by haptens (27). Two concomitant pathways have been identified; one involving activation of the inflammasome, and another involving activation of toll-like receptors (TLRs). These two pathways are illustrated in Fig. 1.

After a hapten penetrates into the epidermis, it induces keratinocytes to produce reactive oxygen species (ROS). One study showed that dose-dependent application of the chemical allergens 2,4,6-trinitrochlorobenzene (TNCB), 4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone, or OXA) and mercaptobenzothiazole induced ROS production both *in vitro* and *in vivo* (28). ROS cause cellular damage, resulting in the release of extracellular ATP, which, in turn, activates the NOD-like receptor protein 3 (NLPR3) inflammasome. Inflammasomes are multiprotein complexes that are able to detect pathogens, irritants, and endogenous danger signals (29, 30). ATP can activate the NLPR3 inflammasome by binding to the transmembrane purinergic receptor P2X₇, located on DCs (31). One study showed that nickel can directly activate the NLPR3 inflammasome independently of ATP, although ROS were still required (32). Activation of the NLPR3 inflammasome activates caspase-1, an enzyme that is responsible for the maturation of the cytokines pro-interleukin (IL)-1 β and pro-IL-18 (27, 31, 33). Keratinocytes produce both IL-1 β and IL-18, and LCs produce only IL-1 β (34, 35). Together, they activate keratinocytes to produce TNF and granulocyte–macrophage colony-stimulating factor (16, 36).

The second pathway involves the extracellular TLRs, which belong to the 'pattern recognition receptor' family. In the epidermis, they can be found on keratinocytes, DCs, fibroblasts, and mast cells. They are able to bind to pathogen-associated molecular patterns, for example lipopolysaccharide (LPS), a bacterial endotoxin, and provide a first line of defence against invading pathogenic microorganisms. Binding of ligands to TLR4 induces the production and secretion of Th1 and Th17 cytokines, among them TNF, IL-1 β , and IL-18 (37). *In vivo* experiments have shown the importance of TLRs in contact hypersensitivity (CHS) the mouse model of human ACD. One study showed that mice lacking both TLR2 and TLR4 did not develop CHS after repeated application of TNCB (38). The role of LPS as a ligand was investigated in germ-free mice (lacking a microbial skin flora) (38). These mice were still able to develop CHS. This led to the notion that the binding of endogenous ligands to TLR2 and TLR4 plays a vital role in the development of CHS.

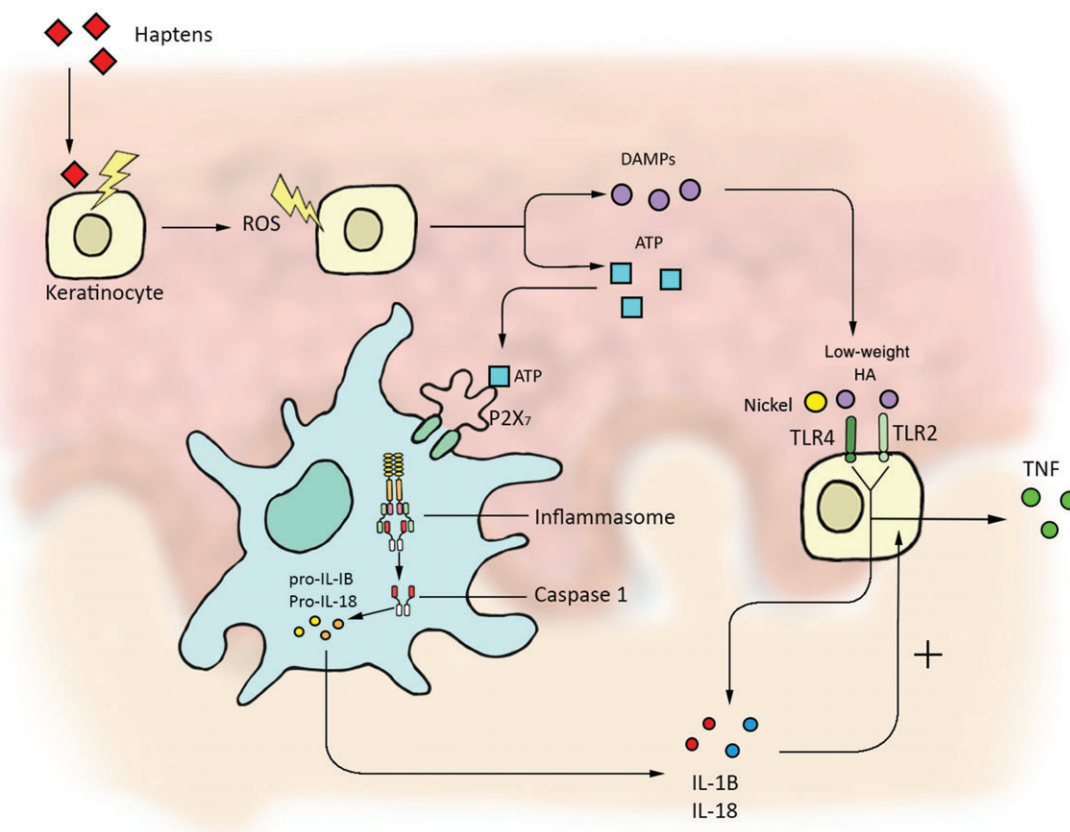


Fig. 1. Activation of the innate immune system after a hapten penetrates the skin. After haptens penetrate the epidermis, they, besides binding to Langerhans cells, activate the innate immune system through a series of steps. First, keratinocytes are induced to produce reactive oxygen species (ROS), which cause cellular damage that leads to the formation of damage-associated molecular patterns (DAMPs) and the release of ATP. ATP activates the NOD-like receptor protein 3 inflammasome by binding to, for example, the transmembrane purinergic receptor P2X₇ on dendritic cells. The activated inflammasome causes caspase-1 activity. Caspase-1 is responsible for the maturation of pro-interleukin (IL)-1 β and pro-IL-18, and subsequently the cytokines IL-1 β and IL-18 are released. At the same time, DAMPs, among them hyaluronic acid (HA) fragments, bind to toll-like receptors (TLR) 2 and 4, on either keratinocytes (as pictured here) or dendritic cells. Nickel and cobalt can bind directly to TLR4. Activation of TLR2 and TLR4 leads to secretion of IL-1 β , IL-18, and tumour necrosis factor (TNF). IL-1 β and IL-18 stimulate keratinocytes to produce more TNF.

Both TLR2 and TLR4 are capable of binding to endogenous ligands, known as damage-associated molecular patterns (DAMPs). In CHS, both oxidative degradation (through ROS) and enzymatic degradation of hyaluronic acid (HA; also known as hyaluronan) contribute to the production of HA fragments (28, 39). HA fragments are proinflammatory molecules, and can act as ligands for TLR2 and TLR4 (40), causing maturation of DCs (41, 42). TNF secreted by keratinocytes is required for HA fragment-induced maturation of DCs (41). The importance of HA fragments and TLR4 in DC migration, and in CHS overall, was further shown in mice overexpressing human hyaluronidase 1 (encoded by *HYAL1*), which showed increased HA degradation (43). Other examples of DAMPs that are TLR4 ligands in CHS are fibronectin extra type III domain A, heparan sulfate, fibrinogen, and

heat shock proteins (44). Besides activation of TLR4 by binding with DAMPs, it has been shown that both nickel and cobalt can bind directly to human, but not to mouse, TLR4 (45, 46).

Besides keratinocytes (the main producers of TNF in the epidermis), more recent evidence suggests that epidermal LCs may also be responsible for increased TNF secretion in CHS (47, 48). Experimental studies showed that CD34⁺ DCs (progenitors of both DCs and LCs) produced TNF when stimulated with certain chemicals (49–51). For example, nickel, oxazolone and hexyl cinnamal increased TNF production in CD34⁺ DCs (49, 50). Conversely, eugenol and 2,4,6-trinitrobenzene sulfonic acid decreased TNF production (50). Besides being hapten-dependent, TNF production by CD34⁺ DCs also showed

individual variation (50). There is also evidence that mast cell-derived TNF plays a role in CD8⁺ DC maturation and migration in CHS (52).

Binding of TNF to TNF-R2 induces maturation of LCs and subsequent migration to draining lymph nodes

There are two TNF receptors: TNF-R1 (or p55TNFR) and TNF-R2 (or p75TNFR). LCs in the skin express TNF-R2 but not TNF-R1 (53). In accordance with this, one group showed that LC migration was normal in TNF-R1 knockout mice when they were challenged with fluorescein isothiocyanate (FITC), but abnormal in TNF-R2 knockout mice when they were injected with TNF (54, 55). When TNF-R2 knockout mice were challenged with oxazolone, they showed a decreased CHS response (54). Another study showed that TNF-R1 also plays an important role in CHS, as TNF-R1-deficient mice did not develop CHS in response to TNCB (56). Further investigation showed that TNF-R1 did not influence DC migration, but increased antigen uptake by DCs (56).

TNF influences LC migration in various ways through binding to TNF-R2. It induces a change in the expression of surface molecules of LCs in the maturation process. Among these changes are: decreased E-cadherin surface expression, and increased expression of CD44, major histocompatibility complex (MHC) II, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and chemokine receptor type 7 (CCR7) (57–60). LCs are positioned in the suprabasal layer of the epidermis, and are bound to the extracellular matrix and keratinocytes through E-cadherin. A decrease in E-cadherin surface expression therefore leads to separation of the LCs (60). CD44 plays an important role in the interaction between LCs and T lymphocytes (61, 62). CCR7-deficient mice show impaired LC migration upon skin painting with FITC (63). One study showed that, although CCR7 expression on maturing LCs is strongly dependent on TNF when they are stimulated with DNCB, it is mostly independent of TNF when they are stimulated with NiSO₄ (64). One study showed that DC stimulation with TNF caused large changes in the actin-based cytoskeleton and highly increased motility (65). In order to reach the lymphatic vessels in the dermis, LCs have to move through the basal membrane. TNF induces LCs to secrete matrix metalloproteinase 9 (also known as gelatinase), which helps to degrade the basal membrane (66).

The role of TNF in the elicitation phase

The elicitation phase of ACD consists of the migration of circulating primed memory/effector T lymphocytes into the (epi)dermis upon re-exposure to an allergen.

The T cells are presented with the hapten by local DCs, and are subsequently reactivated and release proinflammatory cytokines, among them TNF. In order to show the relevance of TNF in the elicitation phase, one group treated sensitized mice with systemic anti-TNF antibodies immediately before challenge with TNCB; the CHS response was reduced significantly (16). Similarly, the CHS response decreased significantly in TNF^{-/-} mice (although it was not absent), and an enhancing role of TNF was postulated (67).

Besides having a cytotoxic effect, TNF has other ways of influencing the elicitation phase. TNF causes increased expression of the adhesion molecules ICAM-1 and VCAM-1, and endothelial leukocyte adhesion molecule (also known as E-selectin), on endothelial cells, increased expression of ICAM-1 and VCAM-1 on DCs, and increased expression of ICAM-1 on keratinocytes (27, 68). These adhesion molecules help the recruitment of circulating effector/memory T lymphocytes into the epidermis. The expression of vascular endothelial growth factor, whose action contributes to oedema by way of increased vascular permeability, was shown to be reduced in TNF^{-/-} mice as compared with wild-type mice (67). TNF also induces IL-33 production in keratinocytes. Blocking IL-33 in mice diminished the CHS response, showing its role in the elicitation phase (69).

Recent discoveries that challenge this model of CHS

Although ample evidence exists to support the role of TNF in inducing LC maturation and migration, more recent evidence suggests that certain allergens, for example 2,4-dinitrochlorobenzene and 2,4-dinitrofluorobenzene (DNFB), can induce LC migration independently of TNF (70). Other investigated allergens, such as oxazolone and *p*-phenylenediamine (PPD), did require TNF, however (70).

The essential role of LCs in CHS was also questioned after studies performed in LC-deficient mice. These studies showed both increased and decreased CHS responses in the absence of LCs (71–73). Despite this absence, the mice were still able to be sensitized, suggesting that dermal DCs are able to mediate a CHS response. The differences in CHS responses seen in these studies performed on LC-deficient mice might be explained by the use of different allergens. Decreased CHS responses were seen in response to TNCB and oxazolone, whereas increased CHS responses were seen in response to DNFB, FITC, and oxazolone (71–73).

One study suggested a tolerogenic role for LCs through their secretion of IL-10 after interaction with CD4⁺ T cells (72). IL-10 influences LC migration negatively, inhibits effector/memory T cells, and induces the development of

regulatory T (T_{reg}) cells (74, 75). One group aimed to clarify this perhaps paradoxical role of LCs, in that they appear both to contribute to, and to counteract, CHS responses. It showed that, at steady state, human skin LCs induce the proliferation of skin-resident T_{reg} cells, thereby mediating tolerance to endogenous antigens (76). However, when LCs were subjected *in vitro* to *Candida albicans*, an increase in the number of antigen-specific memory/effector T cells was seen (76). Another recent study showed how LC migration in response to contact with a weak allergen (2,4-dinitrothiocyanobenzene) led to tolerization *in vivo*. LCs were able to present the hapten to both $CD8^+$ and $CD4^+$ $Foxp3^+$ T_{regs} , leading to deletion or anergy of the former and activation of the latter (25). All in all, the role of LCs in CHS remains controversial (26).

The Single-nucleotide Variants (SNVs) in the Promoter Region of *TNF*

The various SNVs in the promoter region of *TNF*

TNF is located on chromosome 6p21.3, in the MHC III region, situated between the MHC I and MHC II regions, which encode the human leukocyte antigen (HLA) surface molecules (17). Many SNVs* of varying importance have been identified in the promoter region of *TNF* (17). SNVs in the promoter region can lead to either an increase or a decrease in *TNF* mRNA production and *TNF* release by different cell types (17, 78). Interestingly, one study investigated the genetic influence on individual variation in cytokine production, and found this to be ~60% for *TNF* (79).

The SNV in the *TNF* promoter region at location –308 relative to the transcription start site (officially called rs1800629, but also referred to as –308G>A, 308.2, or *TNF2*), in which a guanine (G) is substituted by an adenine (A), was the first SNV to be discovered (80). Many others have since been found, including SNVs at the locations –1031, –863, –857, –575, –376, –238, –163, and –49 relative to the transcription start site (80–82). The SNVs at locations –308 and –238 (rs361525) are the most thoroughly investigated, concerning both function (or pathogenic effects) and their relevance in different diseases. Case–control studies of *TNF* promoter variants in ACD have focused almost solely on the SNV at the –308 location, which we will refer to as *TNF* –308G>A (18–23).

* We have attempted to adhere to the most currently used genetic terminology and nomenclature in this review. Therefore, the term ‘single nucleotide variant’ or ‘SNV’ is used in this article, instead of single nucleotide polymorphism or ‘SNP’. An up-to-date source for genetic nomenclature can be accessed at: <http://varnomen.hgvs.org/> (last accessed on 30-09-2016) (77).

Is *TNF* –308G>A functional?

Whether or not *TNF* –308G>A has a functional effect has been investigated extensively. Investigations have focused on differences in transcriptional activity and/or *TNF* production. These studies have been previously reviewed by different groups, and these have, surprisingly, come to conflicting conclusions (78, 83, 84). We will discuss the different types of studies performed on this topic and the reasons why they might have come to contradictory conclusions.

Promoter activity can be measured *in vitro* by using reporter gene constructs. In this process, the promoter region of interest is attached to a reporter gene, so that it controls the expression of the reporter gene (85). Examples of reporter genes are those encoding chloramphenicol acetyltransferase and luciferase (86, 87). Luciferase is a firefly enzyme that generates light. The expression of the luciferase gene can therefore be quantified by measuring emitted light with a luminometer. By measurement of this light, the influence of SNVs on activity of the promoter region can be investigated. Transcription can be artificially amplified by challenging the reporter gene construct with a stimulant, for example phorbol myristate acetate, LPS, or *TNF* itself. Another *in vitro* method is to measure *TNF* mRNA with an enzyme-linked immunosorbent assay.

There are approximately as many *in vitro* gene reporter construct studies that report significantly increased transcription activity of *TNF* –308G>A as there are that do not show any significant increase or decrease (81, 88–98). A possible explanation for these discrepancies is that different methodologies are used. Some examples of factors that can influence the results of reporter gene assays investigating *TNF* –308G>A are the amount of DNA used, the amount of transfection control vector used, and the method of transfection (98). One group developed a new reporter system that circumvents these influences, and the results suggested that *TNF* –308G>A is functional (98). Another reason for diverging results might be that different cell lines are used to investigate the functionality of *TNF* –308G>A. The first study investigating the functionality of *TNF* –308G>A found a sixfold to sevenfold increase in transcription activity in Raji cells (B lymphocytes) that carry the SNV as compared with the wild type (88). Since then, studies investigating the effect of *TNF* –308G>A in Raji cells, Jurkat cells (T lymphocytes) and U937 cells (pre-monocytes) have found both increased transcription activity and no change in transcription activity. This suggests that the functionality of this variant is perhaps more dependent on the context than on cell type. The choice of stimulant used to amplify transcription can also influence the results.

A second method of investigating the functionality of the *TNF* promoter region variants is to compare differences in *TNF* production between the different alleles/genotypes *ex vivo* or *in vivo* (79, 99–111). These studies have focused on both healthy populations and populations with specific illnesses, for example diabetes (99), multiple sclerosis (101), and sepsis (104, 105, 109). These studies also produced varying results, again most likely because of the varying methods that were used.

Which type of study is able to draw more meaningful conclusions about whether *TNF* –308G>A is functional or not; *in vitro* or *in vivo* studies? One group found that *in vitro* quantification of mRNA was more accurate and sensitive than *in vivo* protein measurement (112). However, if a variant is shown to be functional *in vitro*, this does not necessarily mean that it will be functional *in vivo* (113), as expression of a gene *in vivo* might also be dependent on the tissue type, environmental context, and the genetic background of an individual.

Inheritance of variants in *TNF*

Different variants in the *TNF* promoter region are not inherited independently from each other, or independently of the rest of the MHC complex. Certain alleles within the MHC complex are inherited together as a haplotype. One example of such a haplotype is HLA-A1/HLA-B8/HLA-DR3 (114). An important concept in genetics is linkage disequilibrium. This is the association between a variant and a nearby marker at a population level (115). Alleles that are in close proximity to one another are more likely to be inherited together through linkage disequilibrium. As *TNF* is situated within the MHC III region, alleles in the promoter region are also associated with certain alleles in the MHC I and MHC II regions. The aforementioned haplotype HLA-A1/HLA-B8/HLA-DR3 has been found to be associated with *TNF* –308G>A (114). Certain HLA alleles, for example HLA-DR1, HLA-DR3, and HLA-DR4, have been found to be associated with increased *TNF* secretion by monocytes. Others, however, for example HLA-DR2 and HLA-DR5, have been found to be associated with lower *TNF* secretion (99, 116–118). These findings might be explained by the presence or absence of linkage disequilibrium of HLA alleles with *TNF* –308G>A. Another option is that *TNF* –308G>A is a marker for increased *TNF* production through linkage disequilibrium with an as yet unidentified functional SNV. It is, however, important to keep in mind that linkage disequilibrium is not always necessarily complete; certain alleles might be inherited together often, but not always (119).

The SNVs that have been identified so far in the promoter region of *TNF* have also been found to be associated

with each other, to varying degrees. There are, however, differences between different ethnicities in how frequent the SNVs are and how strong the linkage between them is. One study found that *TNF* –238G>A is often found together with *TNF* –376G>A in people from Kenya, but not in people from Gambia (120). The same association has been found in Caucasians from New England, where the linkage was complete (121). A Dutch study looked at different combinations of *TNF* –238 and *TNF* –308 (together with two alleles in the lymphotoxin α gene) in the Dutch population, and found five different possible haplotypes. In this population, the combination of *TNF* –238G>A and *TNF* –308G>A did not occur (122). Because of these differences between different ethnic groups, it is important to focus on a well-defined homogeneous population when performing genetic association studies, in order to avoid confounding (123).

Investigating the relevance of *TNF* –308A in disease

An appropriate study design for investigating the relevance of an SNV in a specific disease is the case–control design. Some of the requirements for this study design are that the variant under investigation has to have a functional effect, and that the protein produced by its gene plays an important role in the pathophysiology of the disease. In other words, *TNF* –308A carriers have to have a significantly higher level of *TNF* at the site of the disease than *TNF* –308G carriers. The SNV also has to be functional in the cell type responsible for *TNF* secretion in the disease under investigation. Differences can be assessed at either the allele level (comparing –308G carriers with –308A carriers), or at the genotype level, for example by comparing *TNF* –308A/A homozygotes with *TNF* –308G/A and *TNF* –308G/G carriers.

Case–control Studies of *TNF* –308G>A in ACD

There are, to the best of our knowledge, seven studies that have investigated *TNF* –308G>A in ACD (18–24). In the following section, we will take a critical look at these studies to learn from them and gain insights in how to improve future research on this topic. General limitations of these studies are then discussed. Table 1 gives an overview of the methodology of these seven studies, and Tables 2 and 3 show genotype and allele frequencies, respectively.

Westphal *et al.*⁽¹⁾ (18)

This was the first case–control study to focus on genetic variants in ACD. Besides investigating genotype frequencies of *TNF* –308G>A and *TNF* –238G>A, the study also

Table 1. Overview of methodology of studies investigating the TNF- α polymorphism in allergic contact dermatitis (ACD)

Study	Year	Study design	Ethnic group	Patch testing	Contact allergen	Description of controls	Genotyping method
Westphal et al. ⁽¹⁾ (18)	2003	Case-control	(German) 'Caucasian patients'	'Standard series'	Para-substituted aryl compounds	Recruited from blood donor registry	PCR-RFLP
Wang et al. (19)	2007	Cross-sectional	'Taiwanese construction workers'	European baseline series ^a and construction-related substances ^b	Potassium dichromate	Cement workers	PCR-RFLP
Blömeke et al. (20)	2009	Case-control	'Caucasian individuals from Germany and The Netherlands'	No information provided	PPD	'Unrelated Caucasians with no history of sensitization to PPD or ACD'	PCR-hybridization probes
Ertam et al. (21)	2009	Case-control	'Turkish patients'	TRUE Test ^{®c}	Allergens from TRUE Test [®]	No information provided	ASPCR
Khatri et al. (22)	2014	Case-control	Indian population ^d	Indian baseline series ^e	<i>Parthenium hysterophorus</i>	'Healthy individuals' 'recruited from different departments at the All India Institute of Medical Sciences'	ARMS-PCR
Westphal et al. ⁽²⁾ (23)	2016	Case-control	(German) 'white patients'	'Standard series' 'cosmetic preservatives series' 'European standard series'	Allergens from standard series or the cosmetic biocide series	'Healthy unrelated Caucasian blood bank donors'	Real-time PCR
Colagiovanni et al. (24)	2016	Case-control	Not mentioned		Nickel	'Healthy controls' 'selected from the Division of Clinical Nutrition and Nutrigenomics'	PCR

ARMS-PCR, amplification refractory mutation system-polymerase chain reaction; ASPCR, allelic-specific polymerase chain reaction; PCR-RFLP, polymerase chain reaction assay with specific fluorescence-labelled hybridization probes; PPD, *p*-phenylenediamine; TRUE Test[®], Thin-Layer Rapid Use Epicutaneous test.

^aChemotechnique Diagnostic, Vellinge City, Sweden.

^bTrolab, Montreal City, Canada.

^cMekos Laboratories, Hillerød, Denmark.

^dInformation not provided in the article, but a reasonable assumption.

^ePerformed with van der Bend square chambers; includes standardized aqueous extract of *Parthenium hysterophorus*.

Table 2. Overview of the *TNF* –308 genotype frequencies in the case–control studies

Study	Number of individuals		ACD group, n (%)			Control group, n (%)		
	Cases	Controls	GG	GA	AA	GG	GA	AA
Westphal et al. ⁽¹⁾ (18)	86	310	57 (66.3)	26 (30.2)	3 (3.5)	226 (72.9)	84 (27.1)	0 (0.0)
Wang et al. (19) ^a	17	130	11 (64.7)	6 (35.3)	0 (0.0)	113 (86.9)	17 (13.1)	0 (0.0)
Blömeke et al. (20)	181	161	110 (61)	62 (34)	9 (5)	124 (77)	34 (21)	3 (2)
Ertam et al. (21)	50	100	34 (68)	14 (28)	2 (4)	31 (31)	54 (54)	15 (15)
Khatri et al. (22)	60	60	43 (71.7)	13 (21.7)	4 (6.6)	44 (73.3)	16 (26.7)	0 (0.0)
Westphal et al. ⁽²⁾ (23) ^b	239	345	161 (67.4)	69 (28.8)	9 (3.8)	232 (67.3)	104 (30.1)	9 (2.6)
Colagiovanni et al. (24)	41	40	35 (85.4)	5 (12.2)	1 (2.4)	30 (75)	10 (25)	0 (0.0)

ACD, allergic contact dermatitis.

^aOwing to the cross-sectional study design, genotype frequencies were not provided in the original article; the numbers shown in this table are extracted from the data provided in the original article.^bCases are polysensitized individuals.**Table 3.** Overview of the allele frequencies in the case–control studies

Study	Cases		Control	
	A%	G%	A%	G%
Westphal et al. ⁽¹⁾ (18) ^a	18.6	81.4	13.6 ^b	86.5 ^b
Wang et al. (19) ^a	17.65	82.35	7.8	92.2
Blömeke et al. (20)	22.1	77.9	12.4	87.6
Ertam et al. (21)	18	82	42	58
Khatri et al. (22)	17.5	82.5	13.3	86.7
Westphal et al. ⁽²⁾ (23)	18.2	81.8	17.7	82.3
Colagiovanni et al. (24) ^a	8.5	91.5	12.5	87.5

^a Allele frequencies were not mentioned in the original article, therefore, allele frequencies were calculated according to the genotype frequencies with the Hardy–Weinberg principle: $p^2 + 2pq + q^2 = 1$ (and $p + q = 1$), where $p = G$ and $q = A$, and $p^2 = GG$, $2pq = GA$, and $q^2 = AA$.^b Genotype frequencies within this control group deviated from the Hardy–Weinberg equilibrium, and are therefore not representative of the whole population.

investigated genotype frequencies of variants in the genes encoding IL-1 β , IL-1 receptor antagonist, and IL-6. Cases were subdivided into monosensitized and polysensitized individuals. Monosensitization was defined as having a positive patch test (PT) reaction to a *p*-substituted aryl compound (e.g. PPD), and polysensitization as having at least one additional positive PT reaction to a chemically unrelated allergen. Patch testing was performed with a ‘standard series’. Controls were subdivided into individuals with and without a history of eczema. No patch testing was performed in controls. The difference in *TNF* –308G>A genotype distribution between polysensitized cases and controls without eczema was significant ($p = 0.038$), but no significant differences were found in any of the other investigated variants, including *TNF* –238G>A.

The main strength of this study is that it investigated well-defined phenotypes. Polysensitized patients were

compared with healthy controls without eczema, and these phenotypes represent subjects with high and low susceptibility, respectively (124). One limitation is that, in the control group, the *TNF* –308G>A genotype frequencies were not within the Hardy–Weinberg equilibrium, implying that the frequencies of the alleles are not in concordance with those in the general population, and are therefore not fully representative.

Wang et al. (19)

In contrast to the other studies discussed here, this cross-sectional study investigated risk factors for the development of ACD in response to chromate in Taiwanese cement workers. Variants of the *TNF* –308 allele and in the glutathione-S-transferase (*GST*)-*M1* and *GST*-*T1* genes were analysed as risk factors. All subjects underwent patch testing with the European baseline series and construction-related substances. Carrying the *TNF* –308G/A genotype was found to be a significant risk factor for having a positive PT reaction to potassium dichromate [odds ratio (OR) 3.9, CI: 1.14–13.2]. None of the subjects carried the *TNF* –308A/A genotype.

Although this was not a case–control study, and therefore lacked a well-defined control group for comparison, a major strength of this study is that exposure to cement, and therefore to chromate, was equal in all subjects. Because of this, any difference between subjects with a positive PT reaction to chromate and those without can be explained by factors other than exposure, for example by variants in specific alleles. Besides those with allergy to chromate, there were also subjects with positive PT reactions to, for example, ammoniated mercury and nickel sulfate. It is unclear why *TNF* –308G>A was only assessed as a risk factor in chromate-allergic subjects, or what proportion of the chromate allergic-group also had positive PT reactions to other allergens.

Blömeke et al. (20)

This case-control study investigating the association between *TNF* -308G>A and ACD compared patients with a history of ACD and a positive PT reaction to PPD with age-matched and sex-matched controls. It is unclear whether a patient's history of ACD had to be related to PPD exposure. The *TNF* -308A allele was significantly more frequent in cases than in controls (Fisher's two-sided exact test, $p = 0.0016$; logistic regression, $p = 0.0017$), in agreement with the results found by Westphal et al. (18). Analysis of subgroups showed that the association was even stronger in females aged >45 years.

In contrast to the study by Westphal et al., this study did not subcategorize monosensitized and polysensitized subjects, owing to the small number of polysensitized subjects. In comparison, the majority of the population studied by Westphal et al. were polysensitized. Controls had no history of ACD or sensitization to PPD, although it is unclear whether this implies that controls underwent patch testing or not.

Ertam et al. (21)

This Turkish case-control study looked at the association between *TNF* -308G>A and *IL-1RA* variants and ACD. Cases were defined as patients with ACD with a positive PT reaction to one or more allergens in TRUE Test®. More than half of the patients had a positive PT reaction to nickel sulfate. Controls consisted of individuals without a history of eczema and atopy. Interestingly, the frequency of the *TNF* -308G/G genotype was significantly higher in cases than in controls, which is in contradiction to the results found in the previous studies. The proportion of *TNF* -308A/A homozygotes in the control group (15%) is remarkable.

These results might be explained by the different genotype frequencies found in different ethnicities, although the reverse association of the A allele with ACD is still in contradiction to the current theory that the *TNF* -308G>A variant is functional and increases transcription of *TNF* mRNA. If *TNF* -308G>A is only a marker in linkage with the actual disease-causing gene, then the results can be explained by the study population being of a different ethnicity than in previous studies.

Khatri et al. (22)

A case-control study from India investigated the association between *TNF* -308G>A and airborne ACD to the plant *Parthenium hysterophorus*, in which the hapten parthenin, a sesquiterpene lactone, is the relevant allergen (125). The *P. hysterophorus* plant is common in India

and Australia. Cases were defined as patients with ACD caused by *P. hysterophorus*, proven by a positive PT reaction. Cases were only included if they did not have any respiratory allergy and did not receive immunosuppressive drugs. Controls were healthy individuals, not further described. Perhaps surprisingly, no significant difference in genotype frequency between cases and controls was found. This might again be attributable to the difference in ethnicity studied as compared with previous studies in which a significant association with the *TNF* -308A allele was found.

Westphal et al.⁽²⁾ (23)

The second case-control study by the group of Westphal et al. focused on multiple genetic variants in ACD, investigating a total of 15 variants on 13 genes. For the *TNF* gene, only the SNV at location -308 was investigated. Again, cases were subdivided into monosensitized and polysensitized individuals, but these were defined differently; monosensitization was defined as having one or two positive PT reactions, and polysensitization as having positive PT reactions to three or more unrelated allergens. Patch testing was performed with a baseline series and a cosmetic preservatives series. Within the polysensitized group, a 'high-risk' subgroup was selected, which included patients with at least one strong or extremely positive (++ or +++) PT reaction. There was no significant difference in *TNF* -308 genotype distribution between either polysensitized or the 'high-risk' polysensitized cases and controls. The only significantly more frequent variant was the homozygous A/A variant of *CXCL11*, which encodes a chemokine ligand, although this difference was no longer significant after correction for multiple statistical testing.

Colagiovanni et al. (24)

The most recent study investigated the association between *TNF* -308G>A and ACD caused by nickel in 41 cases compared to 40 controls. Patch testing was performed with the European baseline series, and both cases and controls were limited to females aged between 18 and 40 years. Patients with severe chronic disease (including autoimmune diseases) were excluded. There was no significant difference in genotype distribution, except in a small subgroup of polysensitized patients ($n = 4$, with 3 carrying a variant). Polysensitization was, however, not defined, and the 1 patient carrying the rare *TNF* -308A/A genotype had positive PT reactions to nickel, cobalt, and chrome. These positive reactions are more likely to be attributable to either co-exposure or cross-elicitation than to increased susceptibility.

Discussion of the genetic association studies in ACD

Although the studies described above had varying strengths in their design, there were some (common) limitations. One major limitation was that in none of the case–control studies patch testing was performed in the control group, although this might have been done in the study by Blömeke et al. Because of this, subjects with one or more undiagnosed contact allergies might have been included in the control group. Additionally, subjects in the control group might have been highly susceptible to develop sensitization but did not because of low exposure to allergens. None of the case–control studies attempted to assess the level of exposure in its population, because of the difficulty of this task. Exposure assessment would have increased the validity of the studies. Ideally, the level of exposure should be equal in both cases and controls. In general, description and selection of controls was lacking, with only a few inclusion or exclusion criteria being described. Meta-analyses show that the frequency of *TNF* –308G>A is increased in many autoimmune diseases (126, 127). Therefore, subjects with concomitant autoimmune diseases should be excluded, as this could be a confounding factor, and, in most studies, it was unclear whether these patients were excluded or not.

Of the seven studies performed on *TNF* variants in ACD so far, three studies found an association between ACD and *TNF* –308G>A, three found no association, and one found an inverse association. The studies of Westphal et al.⁽¹⁾ and Blömeke et al. are the most similar in ethnicity studied and the allergen to which the cases were allergic to, and both found a positive association (18, 20). However, the second study by Westphal et al., which contained the largest cohort, found no association. Because of these varying results, no definitive conclusion can yet be drawn on whether *TNF* –308G>A is associated with increased susceptibility to develop ACD. Non-replication is a common phenomenon in genetic association studies, and can often be explained by reasons such as small sample sizes, genotyping errors, and population stratification, among others (123, 128).

Future Research on *TNF* Variants in ACD

Basic requirements for a good case–control study

Because of the large number of case–control studies with varying methodological weaknesses, the editors of *Nature Genetics* wrote an editorial about the basic requirements for genetic association studies (129). These include: large sample sizes, small *p*-values, high risk/ORs, associations that are biologically plausible, investigating alleles that have a relevant physiological function, initial studies

accompanied by an independent replication study, and the association being reproducible in both population and family studies. Sample size is one of the most important determinants of quality in case–control studies investigating associations between genotype and disease. Hattersley and McCarthy calculated required sample sizes according to the frequency of the susceptibility allele in controls, and the desired power or OR (128). The frequency of the *TNF* –308A allele in the HapMap CEU population (Utah residents with northern and western European ancestry from the CEPH collection) is ~17.56%, which is approximately the same percentage as was found in the control group in the study by Westphal et al. (2, 23, 130). Hattersley and McCarthy calculated that, for an allele frequency of ~10% in controls, 3066 subjects are needed (or 1533 in each group) to achieve a power of 90% to obtain a *p*-value of 0.05 with an allelic OR of 1.3. For an allele frequency of 20%, the required sample size would be 1772 (or 886 subjects per group). To obtain smaller *p*-values, the sample size required increases even further. The genetic association studies with a focus on ACD performed so far all had relatively small sample sizes.

Phenotype definition

Definition of the phenotype under investigation is another important factor for the quality of a case–control study. Here, we give a suggestion of which phenotype to investigate. There are several different types of selective case–control studies, which differ in the selection of cases and controls (131). One type of selective case–control study is that of extreme discordance, and is especially suited to investigate an association between a specific allele or genotype and disease. In this type, ‘severely affected’ cases are compared with ‘extremely normal’ controls. One suggestion for defining ‘severely affected’ cases among patients with ACD would be polysensitization. The subject of polysensitization as a model of increased susceptibility has been reviewed by Schnuch et al. (124). Polysensitization is defined as being sensitized to more than one allergen. We propose that cases should be polysensitized, with at least three or more positive PT reaction to unrelated allergens in, for example, the European baseline series (132). For controls, we would propose healthy subjects with no history of eczema and negative PT results. Depending on which genes are being investigated, certain concomitant autoimmune diseases should be considered to constitute an exclusion criterion in both groups. Whether these phenotypes are feasible when one is trying to obtain a large sample size might be an issue, and there is no definitive answer to what is better: a small sample size of phenotypically homogeneous

cases, or a large sample size of less well-characterized cases (128). A solution to increase statistical power could be to perform a meta-analysis of multiple studies that have been performed; however, these would need to be sufficiently homogeneous, for example similar phenotypes measured with the same criteria. Current studies differ too much with respect to the investigated ethnicity, the allergens to which the cases are allergic to, and possibly the levels of exposure, for a meta-analysis to be sensibly performed.

Which variant(s) to focus on

It is important that the variant under investigation be both functional and biologically relevant for the studied disease. The main cells believed to be responsible for TNF production and secretion in ACD are keratinocytes, together with epidermal LCs and mast cells (47–52). Studies investigating whether *TNF* –308G>A is functional have looked at pre-monocytes, T lymphocytes, and B lymphocytes, and have obtained conflicting results. It would appear to be important to investigate whether or not this variant is truly functional in keratinocytes and LCs, or in DCs in general.

All studies investigating genetic variants of *TNF* in ACD focused solely on *TNF* –308G>A, except for the study by Westphal et al.⁽¹⁾, which also looked at *TNF* –238G>A (18). This is most likely because *TNF* –308G>A is the most extensively investigated variant in the promoter region of *TNF*. It has the most theoretical support for being functional, as it is located within a binding site for a transcription factor (133). Another option would be to focus on extended haplotypes, including multiple variants in the *TNF* promoter region, as seen in the study by

Bouma et al. (122). However, an important drawback of this approach is that it causes an increase in degrees of freedom with an increasing number of haplotypes, leading to loss of power (115).

Conclusion

TNF plays an important role in the sensitization phase of ACD by inducing LC maturation and subsequent migration to draining lymph nodes. It also plays a role in the elicitation phase by exerting a cytotoxic effect and by promoting migration of circulating primed lymphocytes to the skin. There are many SNVs in the promoter region of *TNF*, of which some are believed to be functional. The most extensively investigated SNV is *TNF* –308G>A. There is no general consensus yet on whether it is functional, a marker for increased TNF production through genetic linkage disequilibrium, or neither. Despite this fact, it has been the focus of many case–control studies in many different diseases and conditions. Seven studies have investigated the association between *TNF* –308G>A and ACD, with varying results. For future studies on this subject, a consensus should be reached on which phenotype to investigate, in order to enable the performance of future meta-analyses, so meaningful conclusions can be drawn. We suggest that polysensitized subjects are compared with healthy controls with preferably comparable exposure, although the feasibility of such a design in terms of sample size remains an issue.

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